

Patent
Attorney's Docket No. 029395-017



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**UTILITY PATENT
APPLICATION TRANSMITTAL LETTER**

Box PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Enclosed for filing is the utility patent application of Joyce TAYLOR-PAPADIMITRIOU; Lukas Carl HEUKAMP; Rienk OFFRINGA; Cornelis Johanna Maria MELIEF; Bruce ACRES; Mireille THOMAS for MUC-1 DERIVED PEPTIDES.

Also enclosed are:

13 sheet(s) of formal informal drawing(s);

a claim for foreign priority under 35 U.S.C. §§ 119 and/or 365 is hereby made to 9921242.5 filed in GB on September 8, 1999; and 99 40 2237.4 filed in EP on September 10, 1999;
 in the declaration;

a certified copy of the priority document;

a General Authorization for Petitions for Extensions of Time and Payment of Fees;

 statement(s) claiming small entity status;

an Assignment document;

an Information Disclosure Statement; and

Other: _____

An executed unexecuted declaration of the inventor(s)
 also is enclosed will follow.

Please amend the specification by inserting before the first line the sentence --This application claims priority under 35 U.S.C. §§119(e) to Provisional Application No. 60/187,215 filed in the United States on March 3, 2000; the entire content of which is hereby incorporated by reference.--



21839

(2/00)

A bibliographic data entry sheet is enclosed.

The filing fee has been calculated as follows and in accordance with the enclosed preliminary amendment:

CLAIMS					
	NO. OF CLAIMS		EXTRA CLAIMS	RATE	FEE
Basic Application Fee					\$690.00 (101)
Total Claims	36	MINUS 20 =	16	x \$18.00 (103)	288.00
Independent Claims	1	MINUS 3 =	0	x \$78.00 (102)	--
If multiple dependent claims are presented, add \$260.00 (104)					
Total Application Fee					978.00
If verified Statement claiming small entity status is enclosed, subtract 50% of Total Application Fee					
Add Assignment Recording Fee if Assignment document is enclosed					
TOTAL APPLICATION FEE DUE					978.00

This application is being filed without a filing fee. Issuance of a Notice to File Missing Parts of Application is respectfully requested.

A check in the amount of \$ 978.00 is enclosed for the fee due.

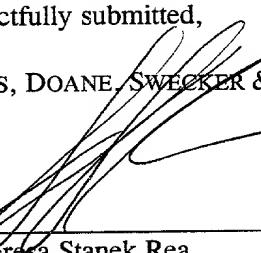
Charge \$ _____ to Deposit Account No. 02-4800 for the fee due.

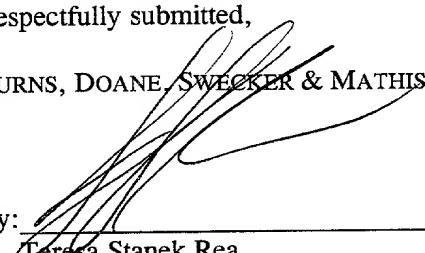
The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§ 1.16, 1.17 and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800. This paper is submitted in duplicate.

Please address all correspondence concerning the present application to:

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Respectfully submitted,


BURNS, DOANE, SWECKER & MATHIS, L.L.P.

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Docket No. 029395-017

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)
Joyce PAPADIMITRIOU et al) Group Art Unit: Unassigned
Application No.: Unassigned) Examiner: Unassigned
Filing Date: September 8, 2000)
For: MUC-1 DERIVED PEPTIDES)

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination, please amend the above-captioned application as follows:

IN THE CLAIMS:

Kindly amend the claims as follows:

Cancel Claim 34.

Claim 1, line 1, delete "consisting of or".

Claim 3, line 1, delete "or 2".

Claim 4, line 1, delete "any one of claims 1 to 3", and insert --claim 1--.

C1-imp 5, line 1, delete "any one of claims 1 to 3", and insert --claim 1--.

git add -A; git commit -m "copy one of claims 5 to 7" and insert --claim 5--.

... "6.1 line 5 to 0", and insert "claim 5--

Claim 11, line 1, delete "any one of".

Claim 12, line 2, delete "as defined in claim 3".

Claim 13, line 1, delete "or 12".

Claim 14, line 1, delete "or 12".

Claim 15, lines 1 and 2, delete "any one of claims 5 to 10 or a vector of any one of claims 11 to 14", and insert --claim 5--.

Claim 16, lines 1 and 2, delete ", such as a mammalian cell".

17. (Amended) A composition comprising a polypeptide of [any one of claims 1 to 3] claim 1, an analogue [of claim 4] thereof, a polynucleotide [of any one of claims 5 to 10], a vector [of any one of claims 11 to 14], comprising said polypeptide or a host cell [of claim 15 or 16] comprising said polypeptide or a combination of two or more of these different compounds.

19. (Amended) [Use of a polypeptide of any one of claims 1 to 3, of an analogue of claim 4, of a polynucleotide of any one of claims 5 to 10, of a vector of any one of claims 11 to 14, of a host cell of claim 15 or 16 or of a composition of claim 17 for the preparation of a medicament] A method for effecting a CTL response in a subject comprising a polypeptide according to claim 1.

Claim 20, line 1, delete "any one of claims 1 to 3" and insert --claim 1--.

21. (Amended) A vaccine comprising a polypeptide of [any one of claims 1 to 3], an analogue [of claim 4] thereof, a polynucleotide [of any one of claims 5 to 10], a vector [of any one of claims 11 to 14] or a host cell [of claim 15 or 16], which vaccine is capable of stimulating a MHC class I restricted T cell response directed to an epitope as contained in a polypeptide of [any one of claims 1 to 3] claim 1.

Claim 23, lines 1 and 2, delete "any one of claims 1 to 3", and insert --claim 1--.

27. (Amended) The product of claim 26 which product comprises (a) an HLA molecule, or a fragment thereof, comprising a polypeptide of [any one of claims 1 to 3] claim 1 or an analogue [of claim 4] thereof in its peptide binding groove, or (b) an analogue of (a) which is capable of inhibiting the binding of (a) to a T cell receptor [of claim 23].

Claim 28, line 1, delete "or 27".

Claim 28, line 2, delete "of claim 23".

Claim 29, line 1, delete "or 27".

30. (Amended) A method of identifying a MHC class I restricted T cell response, said method comprising contacting a population of cells comprising MHC class I restricted T cells with:

the polypeptide of [any one of claims 1 to 3] claim 1 or with [the] an analogue [of claim 4] thereof under conditions suitable for the presentation of the polypeptide or analogue to the T cells,

or

a product [of claim 26 or 27 or cells of claim 29] that selectively binds a T cell receptor;
and

determining whether the CD8 T cells recognize the polypeptide, analogue, the product or the cell, recognition by the T cells indicating the presence of a MHC class I restricted T cell response.

Claim 33, line 3, delete "any one of claims 1 to 3", and insert claim 1.

Claim 35, lines 4-6, delete "any one of claims 1 to 3 with the analogue of claim 4 under conditions in which the polypeptide or the analogue are presented to T cells in the population, or with a product of claim 26 or 27 or with a cell of claim 29", and insert claim 1.

Claim 36, lines 1 and 2, delete "product of claim 26 or 27, a cell of claim 28, the T cell of claim 24 or 25, or a".

Claim 37, line 1, delete "for carrying out a method of any one of claims 30 to 35".

Claim 37, lines 2-3, delete "any one of claims 1 to 3, an analogue of claim 4, a polynucleotide of any one of claims 5 to 10, a composition of claim 17 and/or a product of claim 26 or 27], and insert claim 1 and adjuvants.

REMARKS

Entry of the foregoing amendments is respectfully requested.

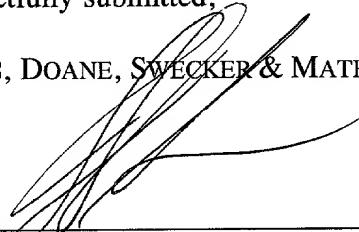
The claims have been amended to eliminate multiple dependency and to place them in better condition for U.S. patent practice.

Should the Examiner have any questions concerning the subject application, a telephone call to the undersigned would be appreciated.

Respectfully submitted,

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By: _____


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Date: September 8, 2000

MUC-1 derived peptides

The present invention relates to MHC class I restricted epitopes and T cells which can be used to prevent or treat cancer or cause immunosuppression; and to the use of the epitopes to diagnose cancer.

In particular, the present invention relates to antigenic polypeptides of the MUC-1 protein which are able to activate Cytotoxic T Lymphocytes (CTL) response and to nucleotide sequences encoding such polypeptides. Furthermore, the present invention relates to vectors comprising such nucleotide sequences, host cells comprising the same and their use in the production of the antigenic polypeptides. In addition, the present invention relates to compositions comprising the polypeptides, nucleotide sequences, vector or host cells of the present invention and to therapeutic and diagnostic uses of such compositions.

Generally speaking, there are two major types of immune response: the humoral response which is characterized by the production of antibodies by B lymphocytes, and the cell-mediated immune response. Antibodies are able to recognize antigens in their three dimensional form, either soluble or bound to an insoluble support such as a cell, while T cells recognize processed antigen fragments which are bound and presented by glycoproteins encoded by the major histocompatibility complex (MHC) notably MHC-I genes which are expressed at the cell surface of almost all vertebrate cells or MHC-II genes which are expressed on antigen presenting cells (APC).

A cell-mediated immune response usually necessitates the cooperation of helper T lymphocytes and effector cells. This cooperation takes place, in particular, as a result of interleukin-2 and/or various other cytokines which are secreted by helper T lymphocytes after their activation by antigenic fragments presented by APC in association with MHC-II. Cytotoxic T Lymphocytes (CTL) are activated, induced to proliferate and to exert their antigen-specific cytotoxic function upon exposure to antigenic polypeptides complexed with

autologous MHC-I, co-stimulatory molecules on the surface of the APC and cytokines, often derived from helper T cells. T cell derived cytokines can also trigger and drive the proliferation and antigen processing capacity of APC as well as activation and induction of proliferation in other cells, including other T cells.

Thus, cytotoxic T lymphocytes (CTL) recognise epitopes bound to MHC class I molecules on the surface of cells. Recognition of such epitopes on the surface of target cells by CTL leads to the killing of the target cells by the CTL. The epitopes which are displayed on the cell are fragments from proteins which have been processed in the class I antigen processing pathway of the cell. In this pathway proteins (generally from the cytoplasm) are broken down in the cytoplasm into small peptides. The small peptides are then transported through the endoplasmic reticulum (where they bind to the MHC molecules) to the surface of the cell.

Said antigen presentation by MHC-I molecules has been characterized (see for example Groettrup et al., 1996, *Immunology Today* 17, 429-435): a full-sized protein or glycoprotein antigen is digested into shorter antigenic polypeptides (of about 7 to 13 amino acids in length). Said polypeptides are associated with MHC-I molecules and β -2 microglobulin leading to a ternary complex which is further presented on the cell surface.

It is not possible to predict which proteins will enter the antigen processing pathway, which fragments will be produced, or which fragments will bind to MHC molecules and be presented at the surface of the cell. Additionally it is not possible to predict which fragments T cells will recognise and whether the T cells which recognise the fragments will be protective.

MHC-I specificity towards antigens can vary greatly depending on the considered MHC-I molecule (HLA-A, HLA-B, ...) and on the allele (HLA-A2, HLA-A3, HLA-A11, ...) since genes encoding the MHC molecules are greatly variable between individuals among a species (reviewed in George et al., 1995, *Immunology Today*, 16, 209-212).

Most tumor cells express antigens at their surface which differ either qualitatively or quantitatively from the antigens present at the surface of the corresponding normal cells. These antigens are specific when they are expressed only by tumor cells. When they are present on both normal and tumor cells, these antigens are said to be associated with the tumor; in this case, they are present either in larger amounts or in a different form in the tumor cells.

It is now well known that patients suffering from a cancer may develop an immune response to their tumor. This has been revealed, in particular, by demonstrating that the serum of some patients contain anti-tumor antigen antibodies, and that their serum was capable of inhibiting

the growth of cancer cells in vitro. Nevertheless, inasmuch as spontaneous tumor regressions are extremely rare, it appears that the immune response observed in vitro remains ineffective *in vivo*.

Hellstrom et al. (1969, *Adv.Cancer Res.*12, 167-223) have shown that antigen-specific CTL can be effective mediators in a tumor-specific immune response. However, this natural immune response is not always effective enough to limit tumor growth. Although an immune response may develop against a tumor, it is not known whether it is of real benefit to the patient. Seemingly uncontrolled tumor growth would suggest that a tumor eludes the body's mechanisms of immune surveillance. Tumor-derived molecules are considered to play a significant part in modifying or diverting the immune response in favor of the tumor rather than in favor of the individual.

In the light of the complexity of the immune response against tumors and the modest state of current knowledge in this field, the use of an anticancer vaccine is not obvious. Animal studies have shown that immunization using living or killed cancer cells could lead to rejection of a subsequent tumor graft, however attempts at immunization using acellular products, for example administration of the complete antigenic protein, with polypeptide fragments of such protein DNA fragment encoding all or part of tumor-associated proteins, have generally been less successful.

Recently, Toes et al. (1997, *Proc. Natl. Acad. Sci.* 94, 14660-14665) have developed an alternative approach based on minimal antigenic polypeptide fragments selection which might be specifically recognized by the CTL. According to said method, the minimal antigenic fragments are expressed in the host cells where they can be associated with MHC-I molecules and then be presented on the cell surface, inducing a specific immune reaction. More specifically, it has been shown that intra-cellular expression of "minigens" encoding very short epitopes (from 7 to 13 amino acids in length) can induce a cellular immune response. Moreover, Whitton et al. (1993, *J. of Virology* 67, 348-352) have proposed the use of a vector, called "string of beads" construct, which co-expresses several minigens and can induce a synergistic CTL immune response.

Another recent and important use for such polypeptides is in association with soluble complexes of MHC-I, β -2 microglobulin and a fluorescent or otherwise visually detectable reagent. These, so called "Tetramers" (*eg*, as described in Altman et al, 1996, *Science*, 274:94-96) can be used to identify by flow cytometry or histology, antigen specific CTL *ex vivo*.

MUC-1 is a glycosylated mucin polypeptide found on the apical surface of mucin-secreting epithelial cells in various tissues, including breast, lung, pancreas, stomach, ovaries, fallopian tubes, and intestine (Peat et al., 1992, *Cancer Res.* 52:1954-60 - Ho et al., 1993, *Cancer Res.* 53:641-51). Malignant transformation of breast, ovary, pancreas and probably other epithelial tissues, results in over expression of MUC-1 polypeptide in tumor cells (Hareuveni et al., 1990, *Eur. Journ. Biochem.* 189:475-86 ; Layton et al., 1990, *Tumor Biol.* 11:274-86). In addition, abnormal glycosylation of MUC-1 polypeptide in breast, and probably other MUC-1-expressing tumour cells results in the exposure of tumor-associated antigenic epitopes on the protein core of MUC-1 (Burchell et al., 1987, *Cancer Res.* 47:5476-82; Devine et al., 1990, *J. Tumor Marker Oncol.* 5:11-26; Xing et al., 1989, *Immun. Cell Biol.* 67:183-95) as well as on the glycosyl side chains (Samuel et al., 1990, *Cancer Res.* 50:4801-8).

Monoclonal antibodies specific for these epitopes have been described which can identify more than 90% of breast and pancreatic tumors. Non major-histocompatibility-complex (MHC) restricted cytotoxic T cell responses to the MUC-1 tumor specific protein epitope by T cells from breast and pancreatic cancer patients have also been reported (Jerome et al., 1991, *Cancer Res.* 51:2908-16) in addition to MHC restricted, MUC-1-specific CTL (Reddish et al., 1995, *Int. J. Cancer* 10:817-823). Moreover, proliferation of T cells to purified MUC-1 has been seen (Keydar et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:1362-6). These various observations suggest that MUC-1 may be an effective target antigen for active immunotherapy in breast, as well as other cancers. Hareuveni et al. (1991, *Vaccine* 9:618-27) expressed the MUC-1 antigen in vaccinia virus and showed that rat immunized with VV-MUC-1 rejected MUC-1-bearing tumor cells at a rate of 60-80 % (Hareuveni et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:9498-502).

The inventors have now identified epitopes which can be used to induce a MHC class I restricted response which is protective against a tumor challenge. The epitopes are from the MUC1 protein. Since activated T cells express MUC1, these epitopes can also be used to induce an immune response against such T cells or be used to obtain products which are capable of targeting activated T cells.

Thus, the present invention concerns immuno-reactive polypeptides identified from the MUC-1 polypeptide sequence and their uses in cancer therapy and diagnosis. The invention could also be used to follow MUC-1 specific immune responses in patients during the course of disease

and/or treatment. The invention also concerns nucleotide sequences encoding these polypeptides, vectors useful for transferring and expressing said nucleotide sequences into target cells, and uses of said nucleotide sequences in cancer gene therapy vaccination and diagnosis.

Accordingly, in a first aspect the present invention relates to polypeptides consisting of or comprising at least one amino acid sequence of at most 20 consecutive amino-acids defined in SEQ ID NO: 1, wherein said polypeptide is different from SEQ ID NO : 2 and is capable of binding with at least one MHC-I molecule.

"Capable of binding with" means that the considered polypeptide is capable to interact and to bind with MHC-I molecules. In a preferred embodiment of the invention, this binding results in cell surface presentation of these polypeptides by MHC class I molecules in order to elicit a specific immune response or for the detection of a specific immune response, eg, by Tetramer analysis (as described, e.g., in Altman et al., 1996, *Science* 274:94-96).

According to a preferred embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO: 3 to SEQ ID NO: 33, SEQ ID NO: 65 and SEQ ID NO: 66. Data to explain why these sequences have been selected are shown in Figures 1 to 7.

According to a preferred embodiment of the invention, said polypeptide presents at least one of the following properties :

- (a) the amino acid sequence is selected from the group consisting of SEQ ID NO:3 to SEQ ID NO:6, SEQ ID NO: 65, SEQ ID NO: 66, and said polypeptide binds the HLA A2 glycoprotein of MHC-I;
- (b) the amino acid sequence is selected from the group consisting of SEQ ID NO:7 to SEQ ID NO: 15, and said polypeptide binds the HLA B7 glycoprotein of MHC-I;
- (c) the amino acid sequence is selected from the group consisting of SEQ ID NO: 16 to SEQ ID NO: 19, and said polypeptide binds the HLA A3 glycoprotein of MHC-I;
- (d) the amino acid sequence is selected from the group consisting of SEQ ID NO: 19 to SEQ ID NO: 21, and said polypeptide binds the HLA A11 glycoprotein of MHC-I;
- (e) the amino acid sequence is selected from the group consisting of SEQ ID NO: 22 to

SEQ ID NO: 25, and said polypeptide binds the HLA A24 glycoprotein of MHC-I;

(f) the amino acid sequence is selected from the group consisting of SEQ ID NO: 26 to SEQ ID NO: 29, and said polypeptide binds the HLA A1 glycoprotein of MHC-I; and

(g) the amino acid sequence is selected from the group consisting of SEQ ID NO: 30 to SEQ ID NO: 33, and said polypeptide binds the HLA B8 glycoprotein of MHC-I.

In a particular preferred embodiment the polypeptide is a peptide that comprises a MHC class I restricted T cell epitope, the epitope being contained in or represented by any one of SEQ ID NO: 1 to 33, 65 or 66, preferably SEQ ID NO: 4, 3, or 5. These latter epitopes lie outside the immunogenic variable non tandem repeat (VNTR) region.

In general, the amino acid sequence present in the polypeptide of the invention may be any stretch of at most 20 contiguous amino acids (such a stretch is in the following referred to as "epitope") of the sequence represented by SEQ ID NO: 1, preferably a sequence represented by any one of SEQ ID NOs: 3 to 33, 65 or 66 below or the amino acid sequence of an epitope present within these sequences (such as the fragments of the sequences shown in the brackets shown below, e.g., for SEQ ID NO: 3 to 5 or for SEQ ID NO: 6, 65 and 66)

SEQ ID NO: 3 - ALGSTAPPV (LGSTAPPV)

SEQ ID NO: 4 - FLSFHISNL (LSFHISNL)

SEQ ID NO: 5 - TLAPATEPA (LAPATEPA)

SEQ ID NO: 6 - SLSYTNPAV (SLSYTNPA or LSYTNPAV)

SEQ ID NO: 65 - LLLTVLTVV (LLLTVLTV or LLTVLTVV)

SEQ ID NO: 66 - ALGSTTPPA (LGSTTPPA)

In one embodiment the polypeptide of the invention has the same sequence as the "epitope". The peptide typically comprises 1, 2, 3 or more copies of each of 1, 2 or more, or all of the above defined "epitopes".

Typically in the polypeptide, a 'linker' sequence may or may not separate the "epitopes" and/or there may or may not be additional (non-"epitope") sequences at the N terminal or C terminal of the polypeptide. Typically the peptide comprises 1, 2, 3 or more linkers. The linkers are typically 1, 2, 3, 4 or more amino acids in length and may comprise amino acid sequence encoded by a polynucleotide sequence that comprises enzyme restriction sites or amino acids

that constitute proteosomal cleavage sites. Thus, in the polypeptide 1, 2 or more, or all of the "epitopes" may be contiguous with each other or separated from each other. The "epitope" sequences may overlap with each other. The polypeptide is typically 8 to 2000 amino acids in length, such as 9 to 1000, 10 to 500, 11 to 200, 12 to 100 or 15 to 50 amino acids.

The peptide may be a natural protein, a fragment thereof, a non-natural protein, or a fusion protein (typically) comprising sequences from different proteins.

The peptide may or may not comprise or be a fragment of MUC1, which fragment may or may not include the MUC1 VNTR. In a preferred embodiment such a fragment only comprises sequences from outside the VNTR. Figure 12 shows a representation of the MUC1 amino acid sequence in which only a single perfect copy of the tandem repeat sequence is shown by amino acids 129 to 148 inclusive. In a particularly preferred embodiment the polypeptide of the invention comprises fragments of the MUC1 sequence shown in Figure 12 or in SEQ ID NO: 2, which fragments do not include any sequence from amino acids 97 to 184, e.g. fragments which do not include sequence from amino acids 90 to 190 (as shown in Figure 12).

SEQ ID NOS: 4, 3 and 5 can be seen at amino acid positions 264 to 272 (inclusive), 167 to 175 (inclusive) and 79 to 87 (inclusive) of Figure 12. In one embodiment the polypeptide only contains a MUC1 sequence that lies close to the epitopes, such as only sequences from or within positions 258 to 276, 253 to 281, 161 to 179, 156 to 184, 72 to 91 or 67 to 96 of Figure 12.

The polypeptide may also comprise a sequence which aids the stimulation of a CTL response directed to the epitope. Such sequence may act as adjuvant or may target the polypeptide to antigen presenting cells (APCs) or to compartments in the antigen processing pathway. The sequence may stimulate a T helper response, such as a Th1 response, and thus may comprise a T helper (e.g. Th1) cell epitope. The polypeptide may also comprise the sequence of any of the proteins mentioned herein.

The polypeptide may be free from modifications. In one embodiment the polypeptide is modified, for example by a natural post-translational modification (e.g. glycosylation) or an artificial modification. Thus, the sequence in the polypeptide may or may not comprise the modification(s) that are present when the sequence is expressed in a normal or cancer cell. The polypeptide may comprise the modifications that occur when it is expressed in a eukaryotic (e.g. human) or prokaryotic (e.g. *E. coli*) cell. In a further embodiment the polypeptide lacks glycosylation.

The modification may provide a chemical moiety (typically by substitution of a hydrogen, e.g. the hydrogen of a C-H bond), such as an amino, acetyl, hydroxy or halogen (e.g. fluorine) group or carbohydrate group. Typically the modification is present on the N or C terminus.

The present invention also relates to analogues of the polypeptides of the invention which are capable of inhibiting the binding of the polypeptide or of an epitope contained in said polypeptide to a T cell receptor either by directly binding to the same T cell receptor or by binding to the same T cell receptor after being processed.

The analogue of the invention is capable of inhibiting the binding of any of the above-mentioned polypeptides (epitopes) to a T cell receptor, either directly or after the analogue is processed. Therefore, certain analogues of the invention can be processed to provide other analogues (that can bind the T cell receptor directly). The term 'analogue' as used here includes both of these types of analogue.

The term 'processed' refers to being processed by the class I antigen presentation pathway (generally this will be hydrolysis, e.g. proteolysis).

Typically the amount of polypeptide (epitope) which can bind the T cell receptor in the presence of the analogue is decreased. This is because the analogue is able to bind the T cell receptor and therefore competes with the epitope for binding to the T cell receptor. The binding of the analogue to the T cell receptor is a specific binding. Generally during the binding discussed above the polypeptide (epitope) or analogue is bound to an MHC class I molecule, such as HLA-A*0201.

The inhibition of binding can be determined using techniques known in the art or any of the techniques or under any of the conditions discussed herein. The T cell receptor used binds specifically to the polypeptide (epitope). T cells with such receptors can be produced by stimulating antigen naive T cells with any of the polypeptides (epitopes) of the invention, for example using the stimulation protocol described in Plebanski et al. (Eur. J. Immunol. 25 (1995), 1783-1787).

Typically an analogue is capable of causing antigen specific functional activation of a T cell which recognises the polypeptide (epitope) (which can be measured using any of the techniques discussed herein). Generally the analogue causes such activation when it is presented to the T cell associated with an MHC class I molecule, such as HLA-A*0201 (for example on the surface of a cell).

The analogue is typically capable of stimulating a MHC class I restricted T cell response directed to the polypeptide (epitope), for example when administered to a human or animal (such as in any of the forms or with any of the adjuvants mentioned herein). Such a response may be protective against a tumor challenge in an animal model or of therapeutic benefit in a human patient.

The analogue typically has a shape, size, flexibility or electronic configuration which is substantially similar to the polypeptide of the invention. It is typically a derivative of the polypeptide.

As well as binding the T cell receptor discussed above the analogue may also be able to bind other specific binding agents that bind the polypeptide (epitope). Such an agent may be HLA-A*0201. The analogue typically binds to antibodies specific for the polypeptide and, thus, inhibits binding of the polypeptide to such an antibody. The analogue is either a peptide or non-peptide or may comprise both peptide and non-peptide portions. Such a peptide or peptide portion may have homology with the polypeptide of the invention.

The analogue may be at least 30% homologous to the polypeptide, preferably at least 50, 70, 80 or 90% and more preferably at least 95%, 97% or 99% homologous thereto, for example over a region of about 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous amino acids. Methods of measuring protein homology are well known in the art and it will be understood by those skilled in the art that in the present context, homology is calculated on the basis of amino acid identity (sometimes referred to as "hard homology"). For example the UWGCG Package provides the BESTFIT program which can be used (e.g. on its default setting) to calculate homology (Devereux et al., Nucl. Acids Res. 12 (1984), 387-395). The homologous peptide typically differs from the epitope present in the polypeptide of the present invention by substitution, insertion or deletion, for example from 1, 2, 3, 4 or more substitutions and/or 1, 2, 3, 4 or more deletions and/or 1, 2, 3, 4 or more insertions over its length. The substitutions are preferably 'conservative'. These are defined according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

Table 1

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
Polar - charged		D E
		K R
AROMATIC		H F W Y

Typically the amino acids in an analogue which has homology with the polypeptide which are equivalent to amino acids in the epitope sequence (such as those which contribute to binding the MHC molecule or are responsible for the recognition by the T cell receptor) are the same or are conservatively substituted.

Typically in the analogue the amino acid in position 2 (based on the numbering used for epitopes bound to MHC molecules) is a L or M and/or the amino acid in position 6 is a V and/or the amino acid in position 9 is V or L.

The analogue (including the homologous peptide discussed above) may comprise 1, 2, 3, 4 or more non-natural amino acids, for example amino acids with a side chain different from natural amino acids. Generally, the non-natural amino acid will have an N terminus and/or a C terminus. The non-natural amino acid may be an L-amino acid.

Typically the analogue is a peptide which comprises one or more modifications. The sequence of the peptide may be the same as the epitope or homologous peptide discussed above. The modification may be any of the modifications mentioned above which can be present on the polypeptide of the invention.

The modification can be present on any of the amino acids of the polypeptide, such as any of the amino acids responsible for binding the MHC molecule or which contact the T cell receptor during recognition by a T cell.

The analogue is typically designed or selected by computational means and then synthesised using methods known in the art. Alternatively, the analogue can be selected from a library of compounds. The library may comprise peptides which have an HLA-A*0201 binding motif.

The library may be a combinatorial library or a display library, such as a phage display library. The library of compounds may be expressed in the display library in the form of being bound to a MHC class I molecule, such as HLA-A*0201.

Analogues can be selected from the library based on any of the characteristics mentioned above, such as their ability to mimic the binding characteristics of the original epitopes. Thus, they may be selected based on the ability to bind a T cell receptor, HLA-A*0201 or antibody which recognises the epitope. They may be selected based on their ability to cause antigen specific functional activity of a T cell that recognises the epitope (for example using any of the techniques or methods of the invention mentioned herein, e.g. CTL assays, ELISPOT assays or by measuring the production of cytokines inside the T cell).

The analogue which is capable of inhibiting the binding of the polypeptide (epitope) to the T cell receptor after being processed is capable of being processed in the class I antigen processing pathway of a cell to provide a second analogue which can directly inhibit the binding of the polypeptide (epitope) to the T cell receptor.

Such a cell is typically a mammalian or avian cell, such as a human or rodent (e.g. mouse or rat cell). The cell may be a muscle cell or a professional APC (such as a dendritic cell, macrophage, Langerhans cell or B cell). Typically the cell will provide the epitope or analogue on its surface bound to a MHC class I molecule.

The invention also relates to polynucleotides comprising a nucleic acid sequence encoding at least one polypeptide or analogue of the present invention. Based on the amino acid sequences provided with in the present application and by using the genetic code those skilled in the art can easily identify said nucleic acid sequences. According to a preferred embodiment, the nucleic acid sequence of the invention is selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 64, and their complementary sequences.

The term "polynucleotide" as used in the scope of the present invention means a DNA and/or RNA fragment, single or double-stranded, linear or circular, natural or synthetic, modified or not (see US-A-5,525,711, US-A-4,711,955, US-A-5,792,608 or EP-A-0 302 175 for modification examples) defining a fragment or a portion of a nucleic acid, without size limitation. It may be, *inter alia*, a genomic DNA, a cDNA, an mRNA. "Polynucleotides" and "nucleic acids" are synonyms with regard to the present invention. The nucleic acid may be in the form of a linear polynucleotide, and preferably in the form of a plasmid. A wide range of plasmids is commercially available and well known by one skilled in the art. These available plasmids are easily modified by the molecular biology techniques (Sambrook et al., 1989, Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

Plasmids derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pBluescript (Stratagene), pREP4, pCEP4 (Invitrogen) and also p Poly (Lathe et al., 1987, Gene 57, 193-201) are illustrative of these modifications. According to the invention, the nucleic acid can be a naked polynucleotide (Wolff et al., Science 247 (1990), 1465-1468) or is formulated with at least one compound such as a polypeptide, preferably viral polypeptides, oligonucleotides or cationic lipids, or cationic polymers which can participate in the uptake of the nucleic acid into the cells (see Ledley, Human Gene Therapy 6 (1995), 1129-1144 for a review) or a protic polar compound (examples are provided below in the present specification or in EP-A-0 890362). "Polynucleotide" also designates nucleic acid of viral origin (viral vector) which encodes at least for the polypeptide of the invention. Such viral vector preferably derives from a virus selected among poxvirus (vaccine virus, MVA, canarypox....), adenovirus, retrovirus, herpes virus, alpha virus, foamy virus or adeno associated virus. Said viral vectors and their uses are widely disclosed in gene therapy literature.

Preferably, said nucleic acid includes at least one therapeutically useful gene sequence that can be transcribed and translated to generate a polypeptide of interest and the elements enabling its expression. The genetic information necessary for expression by a target cell comprises all the elements required for transcription of DNA into RNA and, if necessary, for translation of mRNA into a polypeptide. Transcriptional promoters suitable for use in various vertebrate systems are well known. For example, suitable promoters include viral promoters like RSV, MPSV, SV40, CMV or 7.5k, vaccinia promoter, inducible promoters, tissue specific promoters, synthetic promoters, etc or combination thereof. The nucleic acid can also include intron sequences, targeting sequences, transport sequences, sequences involved in replication or integration. Said sequences have been reported in the literature and can be readily obtained by those skilled in the art. The nucleic acid can also be modified in order to be stabilized with specific components as spermine.

The polynucleotide of the invention is capable of expressing 1, 2, 3 or more (different) compounds, each of which is a polypeptide or analogue of the invention (for example any combination of a polypeptide and an analogue). The polynucleotide is typically DNA or RNA, and is single or double stranded. The polynucleotide generally comprises 1, 2, 3 or more coding sequences which may be the same or different. At least one of the coding sequences

encodes a polypeptide or an analogue of the invention. The coding sequence is typically operably linked to a control sequence capable of providing for expression of the polynucleotide. Thus, typically the polynucleotide comprises 5' and 3' to the coding sequence sequences which aid expression, such as aiding transcription and/or translation of the coding sequence. Typically the polynucleotide comprises a promoter, enhancer, transcription terminator, polyadenylation signal, polyA tail, intron, translation initiation codon or translation stop codon.

The polynucleotide may in particular be capable of expressing a polypeptide or analogue of the invention in a mammalian or avian cell, such as in any of the cells discussed herein. The polynucleotide may furthermore be capable of expressing the polypeptide or analogue in the cellular vector discussed below.

The polynucleotide may form or be part of a vector, such as a plasmid or cosmid vector. In one embodiment the polynucleotide is present in a virus or cellular vector, such as a virus which is capable of stimulating a MHC class I restricted T cell response (e.g. a vaccinia virus).

The introduction or transfer process of an anionic substance of interest into a cell is by itself well known. "Introduction or transfer" means that the polynucleotide is transferred into the cell and is located, at the end of the process, inside said cell or within or on its membrane. It is also called "transfection" or "infection" depending of the nature of the vector.

The invention is therefore further directed to a vector, e.g. of viral or plasmid origin, comprising at least a nucleic acid sequence of the invention.

According to a preferred embodiment, the vector of the invention comprises one or more nucleotide sequences selected from the group consisting of:

- the sequences encoding a polypeptide as defined in (a) in addition to one or more of the sequences encoding a polypeptide as defined in (b), (c), (d), (e), (f) or (g),
- the sequences encoding a polypeptide as defined in (b) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (c), (d), (e), (f) or (g),
- the sequences encoding a polypeptide as defined in (c) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (b), (d), (e), (f) or (g),
- the sequences encoding a polypeptide as defined in (d) in addition to one or more of

- the sequences encoding a polypeptide as defined in (a), (b), (c), (e), (f) or (g),
- the sequences encoding a polypeptide as defined in (e) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (b), (c), (d), (f) or (g),
- the sequences encoding a polypeptide as defined in (f) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (b), (c), (d), (e) or (g), and
- the sequences encoding a polypeptide as defined in (g) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (b), (c), (d), (e) or (f).

Said constructs are named "string of beads" (Whitton et al. 1993, loc. cit.)

Furthermore, the present invention relates to host cells comprising at least one polynucleotide or at least one vector according to the invention. Preferably such a host cell is a prokaryotic cell or a eukaryotic cell, such as a yeast cell, more preferably an animal cell, most preferably a mammalian cell.

The present invention also relates to a composition (iv) that comprises two or more different compounds wherein each of the compounds is (i) a polypeptide or (ii) an analogue or (iii) a polynucleotide of the invention as defined above.

In the composition (iv) 1, 2, 3, 4, 5 or more different compounds may be present, wherein each of these compounds is (i), (ii) or (iii). Thus, the composition may comprise all the epitopes of the invention (present in the form of the polypeptide of the invention), or instead of any of these epitopes the equivalent analogue. The composition may comprise 1, 2, 3, 4, 5 or more polynucleotides which together are capable of being expressed to provide 1, 2, 3, 4, 5 or more different epitopes, analogues or polypeptides of the invention, or all the epitopes (or instead of any of the epitopes the equivalent analogue) of the invention (e.g. in the form of polypeptide of the invention).

In particular, (i), (ii), (iii) or (iv) are provided for use in a method of vaccination against cancer or for use in a method of immunosuppression. 1, 2, 3, 4, 5, or more different epitopes of the invention (or all of the epitopes of the invention) may be used (or instead of any of these epitopes the equivalent analogue). As discussed above if more than one epitope/analogue is used then the combination of epitopes/analogue may be present in the form of the polypeptide of the invention or in the form of the composition of the invention. Similarly 1, 2, 3, 4, 5 or

more different polynucleotides may be used which together are capable of being expressed to provide any of the combinations of epitopes, analogues, polypeptides or compositions mentioned herein.

However, in one embodiment each epitope/analogue or one or more groups of epitopes/analogue within the combination are administered to the host separately or sequentially. The epitopes/analogue in each group are typically together in the form of a single peptide of the invention or in the form of the composition of the invention. Similarly, different polypeptides or polynucleotides of the invention may be administered separately or sequentially, for example polynucleotides capable of expressing individual or groups of epitopes and/or polypeptides and/or analogues and/or compositions.

Thus, the invention provides a combination of 1, 2, 3, 4, 5 or more different epitopes and/or analogues and/or polypeptides and/or compositions and/or polynucleotides of the invention for simultaneous, separate or sequential use in a method of treatment of the human or animal body by therapy, for example in a method of vaccination against cancer or in a method of immunosuppression.

The method of vaccination against cancer or the method of immunosuppression typically leads to a MHC class I restricted T cell response, the T cells of which are specific for an epitope of the invention.

Thus, (i), (ii), (iii), (iv) can be used in a form or manner in which they stimulate such a MHC class I restricted T cell response. Such methods are known in the art. Generally a MHC class I restricted T cell response can be obtained by vaccinating using an appropriate dose, route of administration, adjuvant or delivery system. Thus, the vaccine of the invention may comprise one or more components (for example, as discussed herein in relation to the vaccine of the invention) in addition to (i), (ii), (iii) or (iv). The components of the vaccine may be administered simultaneously, separately or sequentially to the host.

Thus, the invention also provides a vaccine comprising (i), (ii), (iii) or (iv), which vaccine is capable of stimulating a MHC class I restricted T cell response directed to an epitope (polypeptide) of the invention. Typically such a vaccine comprises an adjuvant or delivery system which stimulates a MHC class I restricted T cell response.

The adjuvant may be capable of causing or augmenting a MHC class II restricted T cell (typically CD4) response which is favourable to the production of a MHC class I restricted T cell response, such as a Th1 response. Thus, the adjuvant may comprise a MHC class II

restricted T cell epitope (or a precursor which can be processed *in vivo* to provide such an epitope). The adjuvant may be a cytokine, such as a cytokine which stimulates a MHC class I restricted T cell response or favourable MHC class II restricted T cell response (e.g. IL-2, IL-7, IL-12 or IFN- γ). The adjuvant may be, for example, CFA (Golding and Scott, Ann. N.Y. Acad. Sci. 754 (1995), 126-137), a muramyl dipeptide (e.g. of a mycobacterial cell wall), monophosphoryl lipid A, lipopolysaccharide (e.g. from *B. abortus*), liposomes, SAF-1 (Golding and Scott, Ann. N.Y. Acad. Sci. 754 (1995), 126-137), a saponin (e.g. Quil A), keyhole limpet hemocyanin, yeast TY particle, beta 2-microglobulin or mannan (e.g. oxidised mannan).

The delivery system is typically capable of providing (i), (ii), (iv) or an epitope or analogue expressed from (iii) or (iv) to an APC, such as a professional APC.

As mentioned above the particular route of administration used may aid the stimulating of a MHC class I restricted T cell response and, thus, (i), (ii), (iii), (iv) or the vaccine of the invention may be provided in a form suitable for administering by such a route. Intraperitoneal or intravenous routes are preferred. In one embodiment these substances are delivered by biolistic means.

Generally a low dose of antigen favours the development of a MHC class I restricted T cell response. Thus, in the method a suitable low dose of a compound of the invention can be given. The vaccine may be provided in an amount and concentration that is suitable for administering to provide an appropriate low dose. In one embodiment (iv) is administered in the form of "naked DNA".

The invention also relates to a composition, preferably a pharmaceutical composition, which is particularly useful for the delivery of polynucleotides of the invention to cells or tissues of a subject in the scope of a gene therapeutic method, especially in case of cancer treatment. The term "gene therapy method" is preferably understood as a method for the introduction of a polynucleotide into cells either *in vivo* or by introduction into cells *in vitro* followed by re-implantation into a subject. "Gene therapy" in particular concerns the case where the polynucleotide is expressed in a target tissue, especially tissue comprising cell expressing MHC-I molecules.

Preferably, the composition, in particular pharmaceutical composition, furthermore comprises

a pharmaceutically acceptable carrier or diluent. The carrier or diluent is non toxic to recipients at the dosages and concentrations employed. Representative examples of carrier or diluent for injectable solutions include water, isotonic saline solutions which are preferably buffered at the physiological pH (such as phosphate buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol and ethanol, as well as polypeptides or protein such as human serum albumin. This carrier or diluent is preferably isotonic, hypotonic or weakly hypertonic and has a relatively low ionic strength. Furthermore, it may contain any relevant solvents, aqueous or partly aqueous liquid carriers comprising sterile, pyrogen-free water, dispersion media, coatings, and equivalents. The pH of the pharmaceutical preparation is suitably adjusted and buffered.

The invention more particularly pertains to a composition, in particular pharmaceutical composition, comprising at least one of the complexes described above and also incorporating at least one adjuvant capable of improving the transfection capacity of said complex. Adjuvants may be selected from the group consisting of a chloroquine, protic polar compounds such as propylene glycol, polyethylene glycol, glycerol, EtOH, 1-methyl L -2-pyrrolidine or their derivatives, or aprotic polar compounds such as dimethylsulfoxide (DMSO), diethylsulfoxide, di-n-propylsulfoxide, dimethylsulfone, sulfolane, dimethylformamide, dimethylacetamide, tetramethylurea, acetonitrile and their derivatives.

In a preferred embodiment, the polynucleotide which is contained in the composition is a DNA. Other particular embodiments of the invention are compositions, in particular pharmaceutical compositions, wherein said polynucleotide is naked, associated with viral polypeptides or complexed with cationic components, more preferably with cationic lipids. In general, the concentration of polynucleotide in such a composition is from about 0.1 μ g/ml to about 20 mg/ml.

The composition, in particular pharmaceutical composition, in accordance with the present invention can be administered into a vertebrate tissue. This administration may be made by intradermal, subdermal, intravenous, intramuscular, intranasal, intracerebral, intratracheal, intraarterial, intraperitoneal, intravesical, intrapleural, intracoronary or intratumoral injection, by means of a syringe or other devices. Transdermal administration is also contemplated, as

are inhalation, aerosol routes, instillation or topical application.

According to the present invention, the composition, in particular pharmaceutical composition, can be administered into target tissues of the vertebrate body including those of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, connective tissue, blood, etc. In a preferred embodiment, said composition will be administered into tumor.

Administration of such a composition to a patient allows to elicit an immune response based on the activation of cytotoxic lymphocytes by the polypeptides encoded by said nucleotide sequences. The composition of the invention is particularly suitable for the treatment or prevention of MUC-1-expressing cancers, such as breast cancer, ovary cancer, pancreas or lung cancer.

According to a special embodiment, the compositions of the present invention, i.e. containing polypeptide or polynucleotide sequences of the instant invention (see above), is suitable for the treatment or prevention of MUC1-expressing cancers, wherein said treatment or prevention comprises:

- a step a) consisting in administering to a patient a composition of the present invention,
- a step b) consisting in administering to the same patient in need a second composition, wherein said composition is either a composition of the present invention, or a composition containing a MUC-1 polypeptide, or a polynucleotide encoding such a MUC-1 polypeptide, wherein said MUC-1 polypeptide is the full length MUC-1 polypeptide of SEQ ID NO: 2, or a MUC-1 polypeptide as disclosed in US 4,963,848, US 5,053,489, WO 8805054 or US 5,861,381 corresponding to MUC-1 polypeptide presenting varying tandem repeat sequence.

According to this special embodiment, both administration steps a) and b) can be made independently of each other or in the same time. This special embodiment can result in boosting the immune response developed by the treated patient.

According to the invention, the term "cells" includes prokaryote cells and eukaryote cells, yeast cells, plant cells, human or animal cells, in particular mammalian cells. In particular, cancer cells should be mentioned. The invention can be applied *in vivo* to the interstitial or

luminal space of tissues in the lungs, the trachea, the skin, the muscles, the brain, the liver, the heart, the spleen, the bone marrow, the thymus, the bladder, the lymphatic system, the blood, the pancreas, the stomach, the kidneys, the ovaries, the testicles, the rectum, the peripheral or central nervous system, the eyes, the lymphoid organs, the cartilage, the endothelium. In preferred embodiments, the cell will be a muscle cell, a haematopoietic system stem cell or an airways cell, a tracheal or pulmonary cell, or a tumor cell.

The present invention also relates to a process for transferring a nucleic acid into cells wherein said process comprises contacting said cells with at least one polynucleotide according to the invention. This process may be applied by direct administration of said polynucleotide to cells of the animal *in vivo*, or by *in vitro* treatment of cells which were recovered from the animal and then re-introduced into the animal body (*ex vivo* process). In *in vitro* application, cells cultivated on an appropriate medium are placed in contact with a suspension consisting of polynucleotide of the invention. After an incubation time, the cells are washed and recovered. Introduction of the polynucleotide can be verified (eventually after lysis of the cells) by any appropriate method.

In the case of *in vivo* treatment according to the invention, in order to improve the transfection rate, the patient may undergo a macrophage depletion treatment prior to administration of the pharmaceutical preparations described above. Such a technique is described in the literature (refer particularly to Van Rooijen et al., 1997, TibTech 15, 178-184).

The present invention further concerns the use of a polypeptide or analogue or a polynucleotide, a vector or a host cell as defined above for the preparation of a composition intended for diagnostic, curative, preventive or vaccination treatment of man or animals, and more specifically for the treatment of cancer.

Moreover, the present invention relates to a diagnostic composition comprising at least one polypeptide as defined above. The use of a polypeptide of the invention in a diagnostic composition is illustrated by the following processes:

- a process which enables the detection and eventually the quantification of an antibody directed against said polypeptide consists in (i) contacting with said polypeptide a biological sample susceptible to containing said antibody and (ii) detecting the

formation of an immune complex between said antibody and said polypeptide. a process which enables the detection and eventually the quantification of MUC-1-specific T lymphocytes according to the ELISPOT technique (Scheibenbogen et al., 1997, Clinical Cancer Research 3, 221-226); Tetramer analysis (e.g., as described in Altman et al., 1996, Science 274:94-96) or other techniques which allow the identification of specific T cells by virtue of specificity of their T cell receptor for the polypeptides of this invention.

The methods, compositions, uses of the invention can be used for the treatment of all kinds of cancer the treatment and/or diagnostic of which being related to or dependent on the immune properties of the polypeptides of the invention. The compositions, and uses of the present invention may be desirably employed in humans, although animal treatment is also encompassed by the uses described herein.

The present invention also relates to a T cell receptor which recognizes a polypeptide (epitope) of the invention or a fragment thereof which can recognize the polypeptide (epitope).

The T cell receptor of the invention recognises a polypeptide (epitope) of the invention. The fragment of the T cell receptor typically comprises the extracellular domain. The fragment may be a soluble fragment or a fragment capable of binding to a cell membrane. The T cell receptor or fragment may be modified, such as by any of the modifications described herein in relation to the polypeptide of the invention. The T cell receptor or fragment may be part of a fusion protein.

The T cell receptor or fragment is able to bind an MHC molecule (e.g. HLA-A*0201) that comprises the polypeptide (epitope) in its peptide binding groove. Typically the MHC molecule will be present on the surface of a cell. The T cell receptor or fragment may or may not be able to cause antigen specific functional activity of a T cell upon which they are present. This activity may include cytotoxic activity (such as the killing of the cell that bears the MHC/epitope complex which is recognised) or the secretion of substance (such as IFN- γ) from the T cell. The activity may be measured by CTL assay, ELISPOT assay or by measuring the production of cytokine inside the T cell.

The T cell receptor may be present in a population (or composition) which comprises 2, 3, 4,

5 or more different T cell receptors of the invention which together recognise any of the combinations of a polypeptide or analogue of the invention.

Furthermore, the present invention relates to a T cell which comprises a T cell receptor according to the invention.

Such a T cell is preferably a MHC class I restricted cell, and is typically a CD8 T cell, although in one embodiment it is a MHC class I restricted CD4 cell. Generally when the T cell receptor of the T cell recognises the epitope antigen specific functional activity of the cell occurs (such as the functional activity mentioned above). The T cell may be an antigen naive or antigen experienced T cell. The T cell may be of a cell line, such as an immortalised cell line. The T cell may have been fused with another cell, which may or may not be a T cell.

The T cell is typically obtained from a host, such as a naive host, a host that has cancer or a host that has been immunised with a MUC1 based immunogen, such as any of the polypeptides, analogues or polynucleotides mentioned herein. The T cell may be replicated *in vitro* in an antigen specific (typically by contacting with an epitope or analogue of the invention) or a non-antigen specific manner. Thus, the invention provides a T cell of the invention that has been produced by replication *in vitro*.

The invention also provides a product that selectively binds a T cell receptor of the invention, typically in a reversible manner. Such a product is generally able to inhibit the binding of a polypeptide (epitope) of the invention (e.g. bound to an MHC molecule) to the T cell receptor. The product is typically able to cause antigen specific functional activity of a T cell with the T cell receptor of the invention.

The product typically comprises (a) an MHC molecule, or fragment thereof, comprising a polypeptide (epitope) or analogue of the invention in its peptide binding groove, or (b) an analogue of (a) which is capable of inhibiting the binding of (a) to a T cell receptor of the invention.

The MHC molecule of (a) is generally a class I molecule (e.g. HLA-A*0201). Such molecules comprise an α chain and a β chain. The fragment may comprise only the extracellular domain of the MHC molecule. The fragment may or may not be capable of binding a cell membrane. (b) may comprise a protein which has homology with a naturally occurring α chain (or a fragment thereof) and/or a protein which has homology with a naturally occurring β chain (or a fragment thereof). The naturally occurring α or β chain may be of an HLA-A molecule (e.g.

HLA-A*0201). Any of the above homologous proteins or fragments may be present as part of fusion proteins.

(b) is typically a derivative of (a) and, thus, may be made by modifying (a) by any of the modifications mentioned herein.

The product may be designed, made or identified using methods known in the art. Thus, the invention provides use of a polypeptide (epitope or epitope sequence) of the invention to design or identify the product. The product may be designed by computational means or may be identified from a library of compounds.

Thus, the invention also provides a method of identifying a product of the invention comprising contacting a candidate substance with a T cell receptor or fragment of the invention and determining whether the candidate substance binds to the T cell receptor or fragment, the binding of the candidate substance to the T cell receptor or fragment indicating that the substance is such a product.

In the method the product may be present on the surface of a cell, such as a professional APC. The binding may be measured by contacting the candidate substance with a T cell of the invention and determining whether the candidate substance causes antigen specific functional activity of the T cell (such as by any means mentioned herein).

The product may be linked to a cytotoxic agent. In one embodiment the product is an antibody. In one embodiment 2, 3, 4 or more products are linked together in a multimer and, thus, the invention provides a multimer comprising 2 or more products of the invention. Such a multimer may be used in the same manner as the product is used in the different aspects of the invention and, thus, the term 'product' as used in the context of the other aspects of the invention includes the multimer.

The products in the multimer may be linked by a covalent bond or by non-covalent means. In a preferred embodiment the products are linked by a streptavidin - biotin interaction and, thus, typically the products comprise a biotin portion (typically chemically linked to or in a fusion protein with the product) which allows the products to be linked together by streptavidin.

The multimer generally has a higher binding affinity to the T cell receptor of the invention than the product, and in one embodiment is able to cause more antigen specific functional activity than the product. The multimer may also comprise a detectable label, such as a radioactive or a light detectable (e.g. fluorescent) label. The label may allow the multimer to be sorted by

flow cytometry (e.g. when the multimer is bound to a T cell receptor which is present on a T cell of the invention).

The multimer may be a soluble multimer or may be capable of associating with a cell membrane. In one embodiment the multimer is attached to a solid support, such as a microtitre plate.

The invention also provides a cell comprising a product of the invention. The cell may be any of the types of cells mentioned herein, such as a professional APC or T cell. The cell may be capable of stimulating antigen specific functional activation of a T cell of the invention. Thus, the cell may be used to stimulate a MHC class I restricted T cell response *in vitro* or *in vivo*, which response is directed to a polypeptide (epitope) of the invention. The cell may, therefore, be used in a method of treatment of the human or animal body by therapy, particularly in a method of treating or preventing cancer.

In one embodiment the cell may be made by providing a polypeptide, analogue, polynucleotide or composition of the invention to a cell which is able to process the polypeptide, analogue, polynucleotide or composition and present them on its surface (under conditions in which such processing occurs).

The invention furthermore provides a method of causing the replication of MHC class I restricted T cells which are specific for a cancer epitope comprising contacting a population of cells which comprises MHC class I restricted T cells with a polypeptide or analogue of the invention under conditions in which the polypeptide or analogue are presented to T cells in the population, or with a product or cell of the invention.

The invention includes use of a T cell of the invention (including a T cell replicated by the above method) *in vitro* or *in vivo* to kill a cell which presents the polypeptide (epitope) of the invention. Such a cell is typically a cancer cell, but in one embodiment is a T cell (typically a MUC1 expressing activated T cell). Thus, the invention provides a T cell of the invention, or a cell which has been replicated in the method of the invention for use in a method of treatment of the human or animal body by therapy. In particular for use in a method of preventing or treating cancer or a disease caused by an immune response, such as an inflammatory disorder, autoimmune disease, organ transplant rejection or graft versus host disease.

As mentioned above, the invention also provides a method of identifying a MHC class I restricted T cell response which is based on determining whether MHC class I restricted T cells from a host recognise a polypeptide or analogue of the invention (either of which may be provided by a polynucleotide of the invention), or a product or cell of the invention. In the method the polypeptide or analogue may be in the form of the composition of the invention. In one embodiment the determination of whether the T cells recognise the polypeptide or analogue is done by detecting a change in the state of the T cells in the presence of the polypeptide or analogue or determining whether the T cells bind the polypeptide or the analogue. The change in state is generally caused by antigen specific functional activity of the T cell after the T cell receptor binds the polypeptide or the analogue. Generally, when binding the T cell receptor the polypeptide or the analogue is bound to an MHC class I molecule, which is typically present on the surface of an APC.

The change in state of the T cell may be the start of or increase in the expression of a substance in the T cells and/or secretion of a substance from the T cell, such as a cytokine (e.g. IFN- γ , IL-2 or TNF- α). Determination of IFN- γ expression or secretion is particularly preferred. The substance can typically be detected by allowing it to bind to a specific binding agent and then measuring the presence of the specific binding agent/substance complex. The specific binding agent is typically an antibody, such as polyclonal or monoclonal antibodies. Antibodies to cytokines are commercially available, or can be made using standard techniques.

Typically the specific binding agent is immobilised on a solid support (and thus the method may be based on the ELISPOT assay to detect secretion of the substance). After the substance is allowed to bind the solid support can optionally be washed to remove material which is not specifically bound to the agent. The agent/substance complex may be detected by using a second binding agent which will bind the complex. Typically the second agent binds the substance at a site which is different from the site which binds the first agent. The second agent is preferably an antibody and is labelled directly or indirectly by a detectable label.

Thus, the second agent may be detected by a third agent which is typically labelled directly or indirectly by a detectable label. For example, the second agent may comprise a biotin moiety, allowing detection by a third agent which comprises a streptavidin moiety and typically alkaline phosphatase as a detectable label.

Alternatively, the change in state of the T cell which can be measured may be the increase in

the uptake of substances by the T cell, such as the uptake of thymidine. The change in state may be an increase in the size of the T cells, or proliferation of the T cells, or a change in cell surface markers on the T cell.

The change in state may be the killing (by the T cell) of a cell which presents the polypeptide, the analogue or the product of the invention to the T cell (e.g. the killing of the cell of the invention). Thus, the determination of whether the T cells recognise the peptide may be carried out using a CTL assay.

In one embodiment the T cells which are contacted in the method are taken from the host in a blood sample, although other types of samples which contain T cells can be used. The sample may be added directly to the assay or may be processed first. Typically the processing may comprise diluting of the sample, for example with water or buffer. Typically the sample is diluted from 1.5 to 100 fold, for example 2 to 50 or 5 to 10 fold.

The processing may comprise separation of components of the sample. Typically mononuclear cells (MCs) are separated from the sample. The MCs will comprise the T cells and APCs. Thus, in the method the APCs present in the separated MCs can present the peptide to the T cells. In another embodiment only T cells, (in one embodiment only CD8 T cells), can be purified from the sample. PBMCs, MCs and T cells can be separated from the sample using techniques known in the art.

The T cells used in the assay can be in the form of unprocessed or diluted samples, or are freshly isolated T cells (such as in the form of freshly isolated MCs or PBMCs) which are used directly *ex vivo*, i.e. they are not cultured before being used in the method. However, more typically the T cells are cultured before use, for example in the presence of the polypeptide or the analogue of the invention and, generally, also exogenous growth promoting cytokines. During culturing the polypeptide or the analogue are typically present on the surface of APCs, such as the APC used in the method. Pre-culturing of the T cells may lead to an increase in the sensitivity of the method.

The APC which is typically used in the method is from the same host as the T cell or from a different host. The APC can be a non-professional APC, but is typically a professional APC, such as any of the APCs mentioned herein. The APC may be an artificial APC. The APC is a cell which is capable of presenting the peptide to a T cell. It is typically separated from the same sample as the T cell and is typically co-purified with the T cell. Thus, the APC may be present in MCs or PBMCs. The APC is typically a freshly isolated *ex vivo* cell or a cultured

cell. It may be in the form of a cell line, such as a short term or immortalised cell line. The APC may express empty MHC class I molecules on its surface.

In one embodiment the method identifies a MHC class I restricted T cell response to any of the combinations of a polypeptide or an analogue of the invention discussed above in relation to the composition of the invention. Thus, in the method the T cells can be placed into an assay with the composition of the invention (which comprises the combination of the polypeptide or the analogue which are to be tested). Alternatively, the T cells can be divided and placed into separate assays each of which contains a group of polypeptides or analogues within the combination.

In one embodiment a polypeptide or an analogue *per se* is added directly to an assay comprising T cells and APCs. As discussed above the T cells and APCs in such an assay could be in the form of MCs.

In one embodiment the polypeptide or the analogue are provided to the APC in the absence of the T cell. The APC is then provided to the T cell, typically after being allowed to present the polypeptide or the analogue on its surface. The polypeptide or the analogue may have been taken up inside the APC and presented, or simply be taken up onto the surface without entering inside the APC.

The duration for which the polypeptide or the analogue are contacted with the T cells will vary depending on the method used for determining recognition of the peptide. Typically the concentration of T cells used is 10^3 /ml to 10^9 /ml, preferably 10^5 /ml to 10^7 /ml. In the case where peptide is added directly to the assay its concentration is typically from 0.1 to 1000 μ g/ml, preferably 10 to 100 μ g/ml.

Typically the length of time for which the T cells are incubated with the polypeptide or the analogue is from 4 to 24 hours, preferably 6 to 16 hours.

The determination of the recognition of the polypeptide or the analogue by the T cells may be done by measuring the binding the polypeptide or the analogue to the T cells. Typically, T cells which bind the polypeptide can be sorted based on this binding, for example using a FACS machine. The presence of T cells which recognise the polypeptide will be deemed to occur if the frequency of cells sorted using the polypeptide is above a 'control' value (i.e. above the frequency of antigen naive T cells which recognise the polypeptide or the analogue). The frequency of antigen-experienced T cells during a disease state can be up to 2.5% of the total CD8 T cells.

The polypeptide, the analogue, the polynucleotide, the composition, the product or the cell of the invention can be used to detect a MHC class I restricted T cell response to a polypeptide (epitope) of the invention *in vitro* (such as in a sample from a host) or *in vivo*. This can be done, for example, by using the method discussed above. The presence of a response generally indicates the presence of a cell which is expressing MUC1, such as a cancer cell or an activated T cell. Thus, the detection of the response may be used to diagnose cancer. Measurement of the level of the response may be used to monitor the severity of the cancer (i.e. the number of cancer cells present in the host), a larger response indicating a more severe cancer.

In the method of diagnosis of the invention the presence or absence of the MHC class I restricted T cell response is typically determined by the method of identifying a MHC class I restricted T cell response discussed above.

The antibodies mentioned herein may be produced by raising antibody in a host animal. Such antibodies will be specific to the peptide or to the substances mentioned above which bind antibodies. The peptide or substances are referred to as the 'immunogen' below. Methods of producing monoclonal and polyclonal antibodies are well-known. A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified. A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells, for example as described in Köhler and Milstein (Nature 256 (1975), 495-497).

An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by *in vitro* immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically

acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

Administration

Any of the polypeptides, analogues or polynucleotides discussed above in any form or in association with any other agent discussed above is included in the termed "vaccination agent" below. An effective non-toxic amount of such a vaccination agent may be given to a human or non-human patient in need thereof. The condition of a patient suffering from a cancer can therefore be improved by administration of such a vaccination agent. The vaccination agent may be administered prophylactically to an individual who does not have a cancer in order to prevent the individual developing cancer.

Thus, the present invention provides the vaccination agent for use in a method of treating the human or animal body by therapy. The invention provides the use of the vaccination agent in the manufacture of a medicament for vaccinating against cancer. Thus, the invention provides a method of vaccinating an individual comprising administering the vaccination agent to the individual.

The vaccination agent is typically administered by any standard technique used for administering vaccines, such as by injection.

Typically after the initial administration of the vaccination agent a booster of the same or a different vaccination agent of the invention can be given. In one embodiment the subject is given 1, 2, 3 or more separate administrations, each of which is separated by at least 12 hours, 1 day, 2, days, 7 days, 14 days, 1 month or more.

The vaccination agent may be in the form of a pharmaceutical composition which comprises the vaccination agent and a pharmaceutically acceptable carrier or diluent. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. Typically the composition is formulated for parenteral, intravenous, intramuscular, subcutaneous, transdermal, intradermal, oral, intranasal, intravaginal, or intrarectal administration.

The dose of vaccination may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration and dosage for any particular patient. A suitable dose may however be from 10 μ g to 10g, for example from 100 μ g to 1g of the vaccination agent. These values may represent the total amount administered in the complete treatment regimen or may represent each separate administration in the regimen.

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In the case of vaccination agents which are polynucleotides transfection agents may also be administered to enhance the uptake of the polynucleotides by cells. Examples of suitable transfection agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectamTM and transfectamTM).

When the vaccination agent is a polynucleotide which is in the form of a viral vector the amount of virus administered is in the range of from 10^4 to 10^{12} pfu, preferably from 10^7 to 10^{10} pfu (for example for adenoviral vectors), more preferably about 10^8 pfu for herpes viral vectors. A pox virus vector may also be used (e.g. vaccinia virus), typically at any of the above dosages. When injected, typically 1-2 ml of virus in a pharmaceutically acceptable suitable carrier or diluent is administered.

These and other embodiments are disclosed or are obvious from and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on Internet, e.g. under <http://www.ncbi.nlm.nih.gov/PubMed/medline.html>. Further databases and addresses, such as <http://www.ncbi.nlm.nih.gov/>, <http://www.infobiogen.fr/>, http://www.fmi.ch/biology/research_tools.html, <http://www.tigr.org/>, are known to the person skilled in the art and can also be obtained using, e.g., <http://www.lycos.com>. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation. Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practised otherwise than as specifically described. Accordingly, those skilled in the art will recognize, or able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in

the scope of the following claims.

All of the above cited disclosures of patents, publications and database entries are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication or entry were specifically and individually indicated to be incorporated by reference.

Figures 1 to 7 show competition binding data from polypeptides whose sequences are from within the sequence of human MUC-1. Experiments were performed according to the method described in *van der Burg et al.* (1995, *Hum. Immunol.* 44:189-198). Polypeptides described as 'pp x' correspond to the SEQ ID NO. For example, pp 27 corresponds to SEQ ID NO 27. In some Figures the competition binding curves of some negative (therefore not claimed) polypeptide sequences are shown to demonstrate the specificity of the competition binding assay.

Figures 8, 9 and 10 show ELISpot data from three experiments performed with PBMC, from patients immunized with VV-MUC-1-IL2, exposed to polypeptides from this invention. Spots per 10e6 PBMC indicate the number of CD8+ (CTL) T lymphocytes, per million PBMC, which are specific for that polypeptide. Black histograms represent the ELISpot responses of PBMC drawn from the patient 1 weeks after the injection of VV-MUC-1-IL2 (Figure 8) , 4 weeks after injection (Figure 9) or 4 weeks after the second injection (Figure 10). The white bars correspond to the ELISpot response of patient PBMC taken before VV-MUC-1 administration (Figure 8) 5 months after injection (Figure 9) or before the second injection (Figure 10).

Figure 11 shows that MUC1 derived HLA-A*0201 binding peptides induce peptide specific cytotoxic CTL responses. A2K^b mice were immunised twice with 100µg of MUC1 peptide in IFA and 140µg of Th peptide on day -28 and -14. On day 0 single cell splenocyte suspensions were restimulated *in vitro* for one week with peptide loaded syngeneic LPS-elicited lymphoblasts and tested for cytotoxicity of peptide loaded Jurkat-A*0201K^b. Groups of A2K^b mice were immunised with MUC1²⁶⁴⁻²⁷² (FLSFHISNL), MUC1⁴⁶⁰⁻⁴⁶⁸ (SLSYTNPAV), MUC1¹³⁻²¹ (LLTVLTVV), MUC1¹⁶⁷⁻¹⁷⁵ (ALGSTAPPV) or MUC1⁷⁹⁻⁸⁷

(TLAPATEPA). CTL bulk cultures were tested against Jurkat-A*0201K^b cells loaded with the cognate peptide (filled triangles) or irrelevant influenza matrix control peptide (open circles). Three representative graphs for each peptide are shown. The vertical axis shows % specific lysis.

Figure 12 shows the amino acid sequence of one version of the MUC-1 protein.

The following examples serve to further illustrate the invention.

These examples show:

- 1) The identification of MUC-1 polypeptides which bind specifically to molecules of the human Major Histocompatibility Complex I (MHC-I); and
- 2) The utility of these polypeptides in a functional bioassay, known as ELISpot (Enzyme Linked Immuno Spot) assay.

Example 1

Competition Binding Assay

INTRODUCTION: Polypeptide fragments 8 - 13 amino acids long, of proteins produced within a nucleated, vertebrate cell are associated with newly formed cellular proteins of the MHC-I complex. The complex of MHC-I protein and polypeptide fragment are further associated with a protein known as Beta-2 microglobulin. This trimolecular complex is then transported to the cell surface, anchored to the cell membrane and exposed to the extracellular milieu. Thymus-derived, or T lymphocytes of the CD8 category have specific 'antigen' receptors on their cell surface, which recognise the MHC-I-Beta-2-microglobulin-peptide complex. Individual CD8+ T cells or clonal progeny of an individual T cell precursor express on their cell surface antigen receptors which recognise only one (or very few) such polypeptides within the context of the MHC-I-Beta-2-microglobulin-peptide complex. This is known as 'Antigen Specificity' of T lymphocytes. If the polypeptide is derived from a normal or 'self' protein, T lymphocytes are not 'stimulated' due either to the deletion of self-specific T cells from the immune repertoire or due to negative regulation of self-specific immune responses. If, however, the polypeptide is from a pathogenic organism, such as a virus, then specific T cells are activated to proliferate and to become cytotoxic such that CD8 'cytotoxic

effector cells' or CTL specifically recognise the infected cell and eliminate that cell in an effort to contain the pathogenic condition. Tumours can also produce 'tumour-specific' protein molecules or modifications of cellular proteins. In those cases, specific CD8 T cells can recognise a tumour cell as pathogenic and eliminate these cells by the same mechanism as is used to eliminate virus-infected cells. Often, the tumour-specific modification of a protein is merely quantitative in that a protein is over-produced in tumour cells. It has been shown that such proteins can also be recognised by specific Cytotoxic T Lymphocytes (CTL). For example see Disis *et al.*, *Cancer Research*, 54: 1071-1076 (1994); or Barnd *et al.*, *Proc Natl Acad Sci USA*, 86: 7159-7163 (1989).

It has been shown in numerous publications that the binding of said polypeptides to MHC-I molecules depends upon certain 'motifs' of amino acids at defined positions within the polypeptides. For example, the amino acids Leucine at position 2 and Valine at position 9 of a nine amino acid polypeptide will result in the binding of that polypeptide to HLA-A2. For a review, see Rammensee *et al.*, *Immunogenetics*, 41: 178-228 (1995). The knowledge of the required amino acid positions had been acquired by the extraction of polypeptides from MHC-I molecules and sequencing them. In addition to the 'anchoring' residues there are various other 'preferred' flanking amino acids, such that a polypeptide can be given a 'rank' of likelihood that it will bind to a particular MHC-I molecule, depending on its sequence. Such ranking of predicted binding of polypeptides can be determined by one of several computer programs. In accordance with the invention, the program 'BIMAS' has been consulted (BioInformatics & Molecular Analysis Section) 'HLA POLYPEPTIDE Binding Predictions' (http://bimas.dcrt.nih.gov/molbio/hla_bind/) for predictions of which polypeptides from the human MUC-1 sequence are likely to bind to various HLA types. This, of course, is only a computer prediction and binding must be ascertained with a biochemical assay. Then, whether the polypeptides selected by the binding assay must be tested in a biological assay.

About 200 of the top ranking MUC-1 polypeptides, as predicted by the BIMAS program, predicted to bind to HLA-A1, A2, A3, A11, A24, B7 and B8 were produced (NeoSytem, Strasbourg, France) and were screened for HLA binding by a competitive binding assay. This assay is described in van der Burg *et al.*, *Human Immunology*, 44:189-198 (1995). Briefly, EBV-transformed B lymphocyte cell lines, of a known HLA-type, are exposed to a polypeptide known to bind to that HLA type. Binding of the polypeptide is determined by flow cytometry

using a Fluorescence Activated Cell Sorter. Binding can be envisioned with this apparatus since the polypeptide known to bind is tagged with a fluorescene molecule. Thus cells binding the polypeptide become fluorescent. Each polypeptide to be screened for binding is mixed together with the reference fluorescent polypeptide, which is at a constant concentration of 150 nM. The test polypeptide is added at various concentrations and the mixture exposed to the same cells. A test polypeptide is considered 'positive' if the binding of the reference polypeptide is inhibited by 50 % at 20 μ g/ml or less of the test polypeptide. In Figures 1 - 7 are shown data for the competitive binding of polypeptides deemed, by this assay, to be positive for binding to HLA-A2, B7, A3, A11, A24, A1, and B8 respectively. In each case, binding is compared to a negative control polypeptide, known not to bind to that HLA type and a positive control polypeptide, known to bind to that HLA type. For example, the positive control 'Flu' is a polypeptide with the sequence GILGFVFTL from the influenza virus matrix protein (Scheibenbogen et al. International Journal of Cancer, 71: 932-936 (1997)).

MATERIALS:

EBV-B cell lines were derived by cultivating human Peripheral Blood Mononuclear Cells (PBMC) in filtered culture supernatant which had been used to grow cells from the marmoset line B-958. These cells produce the Epstein Barr Virus. PBMC are cultured for 2-3 days in the presence of 1 μ g/ml Cyclosporin A (to inhibit T cell reactivity to the virus) in the B-958 supernatant, then thereafter cultured in fresh culture medium. HLA-typed EBV-transformed human B cell lines were used for all tests. HLA types are described in Table 2.

Culture medium was Dulbecco's Modified Eagle's Medium (DMEM) + 5x10⁻⁵ M Beta-Mercaptoethanol (with the addition of 25 mM HEPES buffer for the step of polypeptide detachment and cell washing) plus either 2% or 10% Fetal bovine Serum. All tests were performed in 96 well, V-bottom microtitre plates (PS micro plate). β -2 microglobulin was purchased from Sigma. The buffer for the polypeptide detachment used was:

13.76 g citric acid (M MUC-1 210.14 g/L)
 5.43 g Na₂HPO₄. 2H₂O (MMUC-1 177.9 g/L)
 in 500 ml distilled H₂O

pH initially adjusted to pH 4.0, but pH then re-adjusted depending upon which HLA type is

being tested (see Table 2).

Dulbecco's Phosphate Buffered Saline (PBS) was purchased as a powder from Sigma. Reference polypeptides labelled with fluorescene at the cysteine residue (Table 2) were prepared according to Van de Burg et al., 1995, Hum. Immunol., 44, 189-198. Test polypeptides were purchased from NeoSystem, Strasbourg or prepared according to standard methods. Positive control polypeptides were purchased from NeoSystem (Strasbourg, France). Details of the polypeptides used are described in Table 2.

Plastics, unless otherwise indicated, were purchased from Corning.

METHODS:

Cells were cultivated in Corning T175 flasks in 20 of culture medium (10% FBS). The night before the assay, cells were re-suspended and 10 ml of fresh medium added. The day of the test cells were resuspended, counted, pelleted by centrifugation and resuspended in 5 ml complete medium with 10% FBS. Distribute cells into a 6 well plate, 10^5 cells per well in 5 ml culture medium. Cells were then cultured for 4 hours at 37°, 5% CO₂. During that time polypeptides were prepared by dilution in 600 µg/ml in PBS. Two-fold serial dilutions from 600 - 4.68 (to have a final dilution of 100 to 0.78 µg/ml in the test plate) were first prepared in a separate 96 well 'polypeptide dilution' plate.

The test plate (96 well, V-bottom) was prepared as follows:

- negative control (no polypeptide): 50 µl PBS
- positive control (reference polypeptide only): 25 µl PBS + 25 µl Fl-reference polypeptide
- tests: 25 µl Fl-reference polypeptide at 150 nM (final) then 25 µl of test polypeptides (including positive and negative control polypeptides) at their various dilutions were added. Plates were then placed in a refrigerator in the dark.

After the 4 hour incubation of cells, the following were prepared, on ice:

- Two 15 ml conical bottom test tubes containing culture medium with 2% FBS.
- One 15 ml conical bottom test tube containing 10 ml culture medium with 2% FBS and

- including 1.5 µg/ml β-2 microglobulin
- One 15 ml conical bottom test tube containing 2 ml acid 'peptide detachment' buffer at the pH for the particular HLA type as indicated in Table 2.

Cells in the 5 ml culture medium in the 6 well plate were resuspended and transferred to a 15 ml conical bottom test tube and then centrifuged for 5 minutes at 1500 rpm (500g). Resuspend the cells in PBS and centrifuge a second time (500g). Supernatant was removed and 2 ml detachment buffer while on ice. Cells were resuspended by gentle pipetting curing the first 30 seconds of this 2 minute period. After 2 minutes, 14 ml culture medium with 2% FBS was added. Cells are mixed by inverting the tube twice, then centrifuged at 2000 rpm (800g) for 3 minutes at 4°C. Supernatant was removed and cells resuspended in 14 ml cold culture medium with 2% FBS and centrifugation repeated (3 minutes at 800g). Supernatant was removed and cells gently resuspended in 14 ml culture medium, 2 % FBS and 1.5 µg/ml β-2 microglobulin. One hundred µl cells from this suspension were added to each well of the 96 well plate, which already contained the polypeptides. The plate was wrapped in Saran Wrap and left 24 hours at 4°C. The next day, plates were centrifuged at 1000 rpm (200g), supernatant removed and cells resuspended in 100 µl PBS containing 0.1% Bovine Serum Albumin (BSA) and 0.02% sodium azide and cells pelleted by centrifugation at 200g. This step was repeated once more, then cells were resuspended in 1% paraformaldehyde and analysed for fluorescence by a FACScan (Becton Dickenson, Mountainview California).

The mean fluorescence intensity (MFI) of cells with the fluorescent reference polypeptide but with no competitor polypeptide (positive control) was taken as 0% inhibition. Similarly, the MFI of cells without the fluorescent reference polypeptide (negative control) was taken to be equal to 100 % inhibition. Percentage inhibition was calculated as:

$$\% \text{ inhibition} = \left(\frac{(\text{MFI with test polypeptide}) - (\text{MFI negative control})}{(\text{MFI with test polypeptide}) - (\text{MFI positive control})} \right) \times 100$$

High Affinity Binding was taken to be 50 % inhibition at $\leq 10 \mu\text{M}$ polypeptide ($\sim 10 \mu\text{g/ml}$)

TABLE 2: Reference and Positive Control polypeptides used in the Competition Binding assay

Allele tested	elution pH	Reference Polypeptide			Positive Control Polypeptide	B-EBV Line	HLA Type
		sequence (origin)	Conc. pmol/µl	Final Conc. nM			
A1	pH 3.1	YLEPAC*AKY	183	150	CTELKLSDY (<i>Influenza NP 44-52</i>)	MAR	A01, A02, B08, B27, C01, C07
A2	pH 3.1	FLPSDC*FPSV (<i>HBV core 18-27</i>)	250	150	GLLGFVFTL (<i>Influenza matrix 58-66</i>)	JY	A02, B07, C07
A3	pH 3	KVFPCC*ALINK	28 et 20	150	QVPLRPMTYK (<i>HIV nef 73-82</i>)	FRE	A03, A24, B35, B08, C04, C07
A11	pH 3	KVFPCC*ALINK	28 et 20	150		BVR	A11, B35, C04
A24	pH 3.1	RYLKC*QQLL (<i>HIV gp41 583-591</i>)	66 et 20	150	AYGLDFYIL (<i>melanoma p15 10-18</i>)	YT2	A24, B54, C01
B7	pH 3.1	APAPAPC*WPL (<i>human p53 84-93</i>)	29 et 20	150	RPPIFIRRL (<i>EBNA-3A 379-387</i>)	JY	A02, B07, C07
B8	pH 3.1	FLRGRAC*GI (<i>EBNA-3 339-347</i>)	20	150	YLKDQQLL (<i>HIV gp41 591-598</i>)	MAR	A01, A02, B08, B27, C01, C07

Where polypeptide origin is not indicated, HLA-A consensus sequences, as described in Sette *et al.*, Molecular Immunology, 31: 813-822 (1994) were used. C* indicates that the fluorescene molecule is coupled to a cysteine residue (as described in van der Burg *et al.*, Human Immunology, 44: 189-198 (1995)).

Positive control polypeptides are published in Rammensee *et al.*, MHC Ligands and polypeptide Motifs' Springer, New York (1995)

RESULTS:

The competition for binding to selected HLA types between serial dilutions of selected peptides and the reference peptides (as described in Table 2) are shown in Figures 1-7. Binding of polypeptides, from the sequence of human MUC-1, to HLA -A2, B7,A3, A11, A24, A1 and B8 are shown in Figures 1,2,3,4,5,6, and 7 respectively. High affinity binding sequences were often, but not always, within the top 20 predicted binding polypeptide sequences as predicted by the BIMAS HLA Peptide Motif program (as described above).

Example 2

ELISpot

The ELISpot is a technique which allows the identification of antigen-specific (in this case, MUC-1-specific) T cell recognician by the detection of antigen induced production of cytokines (IFN γ , TNF α , IL-4, etc...) following an antigenic stimulation *in vitro*. More particularly, ELISpot allows the determination of the number of antigen specific T lymphocytes in a population of peripheral blood mononuclear cells (PBMC) (Scheibenbogen et al., 1997, Int. J. Cancer 71-1). In this case, the production of IFN γ produced by CD8+ T cells (CTL) in responses to polypeptides as presented by autologous HLA molecules were examined.

Briefly, in an ELISpot, the cytokines are captured between two specific antibodies. The first antibody, specific for human IFN γ , is adsorbed on a nitrocellulose membrane. Lymphocytes from human blood samples are added to the microtitre wells containing the attached antibody. Antigen, in the form of polypeptides, is also added to the wells. The principal is that polypeptides will attach to cell surface HLA molecules (together with β -2 microglobulin). Polypeptide specific T cells will recognize the complex of the polypeptide:HLA: β -2 μ globulin. Upon recognition of antigen, the T cells become 'activated' to produce cytokines such as IFN γ . Secreted IFN γ is then captured by the antibody which is attached to the nitrocellulose. Cells are washed away leaving behind the areas of secreted IFN γ . These areas are revealed by the second antibody (coupled to biotin) and then by a streptavidin-alkaline phosphatase conjugate. The enzyme substrate hydrolysis by the enzyme leads to a spot appearance. Thus each spot

represents the 'fingerprint' of a cytokine producing cell. The tests described below were performed using a commercially available kit (MABTECH, Nacka, Sweden)

MATERIALS

Peripheral blood mononuclear cells :

In Figures 8 and 9 patient PBMC (Peripheral Blood Mononuclear Cells) were obtained from breast cancer patients who had participated in a Phase I clinical trial carried out in the Institut Curie, Paris. In Figure 10, patient PBMC came from prostate cancer patients who had participated in a similar immunotherapy Phase I trial in Los Angeles, USA. In these trials, patients were immunized with a Vaccinia virus construct which expresses, upon infection, the both MUC-1 and IL2. The goal was to generate an immune response to MUC-1 which is an antigen over-expressed on both types of cancers. PBMC were isolated from peripheral blood by Hypaque-Ficol density centrifugation and resulting mononuclear cells frozen in aliquotes of 2 to 4×10^6 cells in a 1 ml volume of culture medium containing 10% DMSO and stored in the vapour phase of liquid nitrogen until use.

TABLE 3

Patient Identification Code	Cancer	HLA-Type
Pt#4 SOM	Advanced Breast	HLA-A 01/02; B 44/08
Pt#5 LEC	Advanced Breast	HLA-A 02/24; B 07/44
A002	Prostate	HLA-A01/26; B38/08

Tissue typing :

Breast cancer patient PBMC was HLA typed by serology and PCR at the Etablissement de Transfusion Sanguine, Strasbourg. Prostate cancer patients' PBMC were HLA typed by PCR at Transgene using the 'One Lamda' HLA typing kit (One Lamda, Canoga Park, CA, USA)

Polypeptides :

Polypeptides were produced at NeoSytem (Strasbourg, France)

ELISPOT :

The ELISPOT kit was purchased from and used according the instructions of MABTECH (Nacka, Sweden). The technique was carried out according to the manufacturer's instructions. Briefly, PBMC were cultured in 96 well microtitre plates for 48 hours in the presence of test or control polypeptides at 5 µg/ml and recombinant IL-2 at 30 units/ml. IFN γ spots were revealed with a second antibody reagent, also specific for Human Interferon gamma, according to the manufacturer's instructions.

RESULTS:

The results from three experiments are shown in Figures 8, 9 and 10. Patient PBMC were taken from liquid nitrogen and thawed the day before the ELISpot assay. The controls and the polypeptides (numbered according to their SEQ ID N°) were added as described above. Duplicate or triplicate wells containing 1- 2 x 10⁵ PBMC were used. The number of spots was determined and is represented as the number of spots per 10⁶ cells.

These data shown in Figure 8 show that PBMC from patient #4 (who is HLA-A2) are able to respond to the polypeptide of SEQ ID NO: 4 in that PBMC from this patient are stimulated to produce IFN γ in response to the presence of this polypeptide but not in the presence of the negative control polypeptide or polypeptide SEQ ID N° 3. The response is seen after vaccination (black histograms), but not before (white histograms). In Figure 9 is shown the results of an experiment in which PBMC from patient #5 (HLA-A2 and B7) are stimulated to produce IFN γ ELISpots upon exposure to polypeptide 4 (SEQ ID N° 4) and polypeptide 10 (SEQ ID N° 10) but not to the negative control or to polypeptides 3 or 7. No PBMC from prior to vaccination were available, but the patient's T cell response, as determined by *in vitro* CD4+ T cell proliferation assay, to a longer (24 aa) MUC-1 polypeptide was discernable only in the weeks following vaccination but was undetectable 5 months afterwards. The transient nature of the T cell responses is verified in Figure 9 in that only PBMC taken 28 days after

vaccination (black histograms) were able to produce ELISpots over background, whereas PBMC taken 5 months after injection produced no ELISpot response to these peptide (white histograms).

These examples demonstrate the value of the invention in the diagnosis of a CD8+T cell immune response to MUC-1.

The invention could also be used in other diagnostic applications such as Tetramer analysis in which soluble MHC-I, beta-2-microglobulin and polypeptides of this invention are complexed together with a fluorescent reagent. The complex is then used to fluorescently tag T cells with an antigen receptor specific for that polypeptide. The quantification of the specific T cells is accomplished with a fluorescence activated flow cytometer and can be done by one practised in the art.

The polypeptides of the invention could also be used in therapeutic or vaccine composition in order to prevent or treat MUC-1-expressing cancers. Polypeptides could be administered alone or complexed with MHC-I and beta-2-microglobulin to stimulate a MUC-1-specific CD8+ (CTL) T cell immune response. The invention could also be used as a DNA-based vector in which oligonucleotide sequences coding for the polypeptides of this invention, incorporated into viral or synthetic vector, are used to vaccinate a patient for the treatment or prevention of MUC-1-expressing cancers.

Example 3

Prediction of peptides that bind HLA-A*0201

A computer program (D'Amaro et al., *Hum. Immunol.* 43 (1995), 13-18) was used to scan the MUC1 sequence with two tandem repeats for nine amino acid long peptides complying with the anchor residue motifs for HLA-A*0201. A full set of ninemers with an eight amino acid overlap covering the tandem repeat as well as ninemers in the top 10% of the scoring data for HLA-A*0201 were synthesised (90 peptides in total) by fmoc chemistry with a yield of 5-15 mg.

Example 4

Testing the synthesised peptides in a binding assay

Peptide binding to HLA-A*0201 was analysed using HLA-A*0201⁺ B lymphoblastoid JY cells in a semi-quantitative competition assay (van der Burg et al. (J. Immunol. 156 (1996), 3308-3314)). The assay is based on competitive binding of two peptides to acid stripped MHC class I molecules on a B cell line (JY). A test peptide competes with a fluorescently labelled reference peptide for the empty class I molecules on the cell surface. Mild-acid-treated JY cells were incubated with 150nM fluorescein (FL)-labelled reference peptide FLPSDC(-FL)FPSV and with several concentrations of competitor peptide for 24 hours at 37°C in the presence of 1.0µg/ml β2-microglobulin. Subsequently, the cells were washed, fixed with paraformaldehyde and analysed by flow cytometry. The mean fluorescence (MF) obtained in the absence of competitor peptide was regarded as maximal binding and equated to 0%; the MF obtained without reference peptide was equated to 100% inhibition. The percentage inhibition was calculated using the formula:

$$\{1 - (\text{MF 150nM reference and competitor peptide} - \text{MF no reference peptide}) / (\text{MF 150nM reference peptide} - \text{MF no reference peptide})\} \times 100\%$$

The binding capacity of competitor peptides is expressed as the concentration needed to inhibit 50% of binding of the FL-labelled reference peptide (IC₅₀). All peptides were tested several times in two fold dilutions starting with a concentration of 100µM. The six peptides that showed any significant binding were further analysed. The IC₅₀ values of these peptides are shown in the table below together with the value for a flu peptide.

The peptides are defined in terms of the amino acid numbering used in Figure 12. The tandem repeat can be defined using the restriction enzyme SmaI which cuts at CCCGGG three times in the MUC1 sequence, once either side of the tandem repeat and once in the C-terminus. This leads to the tandem repeat being defined as amino acids 129 to 148 in Figure 12. The six peptides were analysed further as described below.

Peptide Position	Amino Acid Sequence	Motif Score *	IC ₅₀ mM/ml
Flu Matrix ⁵⁸⁻⁶⁶	GILGVVFTL	54	<5
MUC1 ²⁶⁴⁻²⁷²	FLSFHISNL	59	3-5
MUC1 ⁴⁶⁰⁻⁴⁶⁸	SLSYTNPAV	62	5-10
MUC1 ¹³⁻²¹	LLLTVLTVV	63	6-10
MUC1 ¹⁶⁷⁻¹⁷⁵	ALGSTAPPV	64	10
MUC1 ⁷⁹⁻⁸⁷	TLAPATEPA	58	10-15
MUC1 ¹⁰⁷⁻¹¹⁵	ALGSTTPPA	56	25

* The algorithm used to define the motif score is described in (D'Amaro et al. (loc. cit.)).

Example 5

Testing the peptides in a cytotoxic T lymphocyte (CTL) assay

Summary of assay

To show that the six peptides were functional *in vivo*, transgenic mice expressing the chimeric protein A*0201K^b (Vitiello et al. (J. Exp. Med. 173 (1991), 1007-1015)) underwent an immunization protocol with a MUC1-derived peptide and T helper epitope formulated with adjuvant. The mice were then sacrificed and the splenocytes restimulated by culturing with peptide-loaded, irradiated LPS-elicited B lymphoblasts. The restimulated cells were separated from the lymphoblasts and used in a CTL assay as effector cells. Effector cells were incubated with Na⁵¹CrO₄ loaded target cells at various E:T ratios and cell killing estimated by measuring the amount of ⁵¹Cr released into the cell supernatant using a gamma radiation counter.

Immunisation of mice with MUC1-derived peptides

Transgenic mice expressing the chimeric protein A*0201K^b (Vitiello et al., loc. cit.) were immunised subcutaneously in the base of the tail with 100µg of MUC1-derived peptide and 140µg of H-2 I-A^b-restricted HBV core antigen-derived T helper epitope (amino acid sequence; TPPAYRPPNAPIL) (Milich et al., Proc. Natl. Acad. Sci. USA 85 (1988), 1610-1614) emulsified in a 1:1 ratio with Incomplete Freund's Adjuvant (IFA) in a total volume of 200 µl. After a minimum of two weeks, the mice were boosted using the same protocol.

Preparation of LPS-elicited B lymphoblasts

Splenocytes from (unimmunized) transgenic mice expressing the chimeric protein A*0201K^b (Vitiello et al., loc. cit.) were prepared 72h prior to use as stimulator cells. The cells of several mice were pooled and resuspended in IMDM N medium (IMDM (Biowhittaker) supplemented with 2mM L-glutamine, 8% (v/v) heat inactivated foetal calf serum (FCS), 20µM 2-mercaptoethanol and 100 IU/ml penicillin) containing 25µg/ml LPS (Sigma) and 7µg/ml dextran sulphate (Pharmacia). A 30 ml culture of cellular concentration, 1.5 X 10⁶ cells per ml was incubated at 37°C for 72h.

Cells were then collected, resuspended in IMDM N, separated on a Ficoll gradient and adjusted to a cellular concentration of 5×10^6 cells/ml. Cells were then irradiated for 8 min (the equivalent of 2500 RAD). Cells were then washed once and resuspended in IMDM to a cellular concentration of 40×10^6 cells/ml.

Each MUC1 derived peptide, at a concentration of 100 µg/ml, was incubated for 1h at 37°C with 1 ml LPS-elicited B lymphoblasts. The cells were then washed once and resuspended in IMDM N at a concentration of 10×10^6 cells/ml.

Restimulation of splenocytes from peptide-immunized mice

Two weeks after the final immunization, the mice were sacrificed and the spleens removed. Splenocytes (30×10^6 cells in a 9 ml volume of IMDM N medium) were restimulated by incubation in complete medium with a 1 ml volume of syngeneic, irradiated LPS-elicited B cell lymphoblasts (such that the ratio of splenocytes to blast cells is 3:1). On day 7 of culture the cells were separated on a Ficoll gradient, resuspended in IMDM N medium and counted to generate a preparation of effector cells of known concentration.

Preparation of target cells

The Jurkat-A*0201K^b cell line which is a stable transfectant of a human T cell leukaemia line expressing the product of the HLA-A*0201K^b chimeric gene construct was used as a source of target cells.

Cells growing in log phase were harvested, washed once, counted and 10^6 cells transferred to a microfuge tube. The cells were pelleted and resuspended in a $100\mu\text{l}$ volume of 1 mCi/ml $\text{Na}^{51}\text{CrO}_4$ solution (Amersham) followed immediately by the addition of a $5\mu\text{l}$ volume of 1M HEPES pH 7.0 and gentle mixing of the cell suspension by pipetting. The tubes were incubated

for 1h at 37°C. The cells were then washed four times in IMDM N medium and resuspended in a 25 ml volume of IMDM N medium containing the relevant peptide. After a 20 min incubation the cells were plated out into wells already containing effector CTLs. The final concentration of peptide in each well was 2 μ g/ml.

^{51}Cr release assay

Effector cells, prepared as above, were added in triplicate to wells of a 96 well plate (round bottom wells) such that the resulting ratio of Effector:Target cells was a range from 5:1 to 100:1. For each target cell line tested, six wells containing IMDM N or PBS with 2% (v/v) triton X-100 were prepared as controls to measure the spontaneous and maximal release of ^{51}Cr respectively.

A 50 μ l volume of the preparation of target cells (1000 or 2000 cells depending on preparation and number of effector cells) was then added to each well and the 96 well plates centrifuged for 2 min at 1200rpm. The plates were then incubated for 6 h at 37°C. The culture supernatants from each well were then harvested using Skaton harvesting frames according to the manufacturer's instructions and the ^{51}Cr in each supernatant measured using a Wallac gamma counter.

The data was presented as %age specific ^{51}Cr release which is defined as $100 \times ([\text{experimental cpm} - \text{spontaneous cpm}] / [\text{total cpm} - \text{spontaneous cpm}])$ where the experimental value was the average of three test wells, the spontaneous value, the average of six wells containing IMDM N and target cells and the total value is the average of six wells containing 2% (v/v) triton X-100 and target cells. Data is shown for peptides MUC1⁷⁹⁻⁸⁷, MUC1¹⁶⁷⁻¹⁷⁵, MUC1²⁶⁴⁻²⁷², MUC1⁴⁶⁰⁻⁴⁶⁸ and MUC1¹³⁻²¹ in Figure 11.

Example 6

Protection assay

Mice were inoculated subcutaneously with 10^5 , 5×10^5 and 10^6 B16-MUC1-A2K^b cells (a melanoma cell line constitutively expressing MUC1 and the chimeric gene product HLA-A*0201K^b). Tumour growth was observed 20 days post inoculation and continued until sacrifice of the animal. An inoculation of 5×10^5 B16-MUC1-A2K^b was defined as the optimal

dose for tumour challenge experiments.

To test whether the HLA-A*0201 binding peptides that were previously identified could protect A2K^b transgenic mice (Vitiello et al., loc. cit.) against subsequent tumour challenge with B16-MUC1-A2K^b, groups of 6-8 animals were immunised with 100 μ g of peptide in IFA in the presence of 140 μ g of the H-2 I-A^b-restricted HBV core antigen-derived T helper epitope (128-140; sequence TPPAYRPPNAPIL)(Milich et al., loc. cit.), on day -28, boosted on day -14 and challenged with 5 \times 10⁵ B16-MUC1-A2K^b cells on day 0. Control mice were given IFA or PBS. A measurable tumour was defined as having a volume greater than 36 mm³.

Results from these experiments are shown in the tables below in the form of the percentage of mice surviving at a given day. For experiments 2 and 3 results using a vaccinia construct that expresses MUC1 (VV-MUC1) are also shown. In other experiments immunising with MUC1¹⁶⁷⁻¹⁷⁵ and boosting with MUC1⁷⁹⁻⁸⁷ or immunising with MUC1⁷⁹⁻⁸⁷ and boosting with MUC1¹⁶⁷⁻¹⁷⁵ gave a percentage survival of between 60 and 70% at day 30. Experiment 3 shows results from an experiment in which the mice were inoculated with 8 \times 10⁵ A2K^b dendritic cells (DC) which had been pulsed with the peptides.

Experiment 1

Day	0	21	22	23	26	32	34
IFA	100	38	0	0	0	0	0
MUC1 ²⁶⁴⁻²⁷²	100	75	75	75	63	63	63
MUC1 ¹⁶⁷⁻¹⁷⁵	100	63	63	63	63	63	63
MUC1 ⁷⁹⁻⁸⁷	100	100	75	75	75	75	63
MUC1 ⁴⁶⁰⁻⁴⁶⁸	100	25	25	25	25	25	25
MUC1 ¹³⁻²¹	100	25	13	13	0	0	0
VV-MUC1	100	75	75	75	63	63	38

Experiment 2

Day	0	13	24	26	28	33	38	46	52	53
IFA	100	100	70	50	50	40	40	30	30	30

MUC1 ²⁶⁴⁻²⁷²	100	100	100	88	88	75	75	63	63	63
MUC1 ¹⁶⁷⁻¹⁷⁵	100	100	88	63	63	63	63	38	38	38
MUC1 ⁷⁹⁻⁸⁷	100	100	100	100	100	88	88	75	75	75
MUC1 ⁴⁶⁰⁻⁴⁶⁸	100	100	100	75	75	50	50	38	38	38
MUC1 ¹³⁻²¹	100	100	50	25	25	25	25	25	25	25
VV-MUC1	100	100	90	80	80	80	80	60	60	60

Experiment 3

Day	0	15	21	24	27	32	39	40	42	45	72
PBS	100	89	56	11	11	11	11	11	11	11	0
DC + Flu Matrix ⁵⁸⁻⁶⁶	100	88	63	50	50	38	38	38	38	38	38
DC + MUC1 ²⁶⁴⁻²⁷²	100	100	88	88	88	88	88	88	88	88	88
DC + MUC1 ¹⁶⁷⁻¹⁷⁵	100	100	78	78	78	78	78	78	67	67	67
DC + MUC1 ⁷⁹⁻⁸⁷	100	100	89	89	89	67	67	67	67	67	67
DC + MUC1 ⁴⁶⁰⁻⁴⁶⁸	100	100	75	63	38	38	25	25	25	13	13
DC + MUC1 ¹³⁻²¹	100	100	67	56	56	44	22	22	22	22	22

CLAIMS

1. A polypeptide consisting of or comprising at least one amino acid sequence of at most 20 consecutive amino-acids defined in SEQ ID NO: 1, said polypeptide binding at least one MHC-I glycoprotein, with the proviso that said polypeptide is different from SEQ ID NO: 2.
2. The polypeptide of claim 1, wherein the amino acid sequence is selected from the group consisting of the amino acid sequences shown in SEQ ID NO: 3 to SEQ ID NO: 33, SEQ ID NO: 65 and SEQ ID NO: 66.
3. The polypeptide of claim 1 or 2, wherein the amino acid sequence is selected from the group consisting of:
 - (a) SEQ ID NO: 3 to SEQ ID NO: 6 and SEQ ID NO: 65 and SEQ ID NO: 66, and said polypeptide binds the HLA A2 glycoproteins of MHC-I;
 - (b) SEQ ID NO: 7 to SEQ ID NO: 15, and said polypeptide binds the HLA B7 glycoproteins of MHC-I;
 - (c) SEQ ID NO: 16 to SEQ ID NO: 19, and said polypeptide binds the HLA A3 glycoprotein of MHC-I;
 - (d) SEQ ID NO: 19 to SEQ ID NO: 21, and said polypeptide binds the HLA A11 glycoproteins of MHC-I;
 - (e) SEQ ID NO: 22 to SEQ ID NO: 25, and said polypeptide binds the HLA A24 glycoproteins of MHC-I;
 - (f) SEQ ID NO: 26 to SEQ ID NO: 29, and said polypeptide binds the HLA A1 glycoproteins of MHC-I; and
 - (g) SEQ ID NO: 30 to SEQ ID NO: 33, and said polypeptide binds the HLA B8 glycoproteins of MHC-I.
4. An analogue of the polypeptide of any one of claims 1 to 3, which is capable of inhibiting the binding of the polypeptide or of an epitope contained in said polypeptide to a T cell receptor either by directly binding to the same T cell receptor or by binding to the same T cell receptor after being processed.

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5. A polynucleotide encoding the polypeptide of any one of claims 1 to 3.

6. The polynucleotide of claim 5, comprising a nucleotide sequence selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 64, and their complementary sequences.

7. A polynucleotide encoding the analogue of claim 4.

8. The polynucleotide of any one of claims 5 to 7, further containing elements allowing the expression of the polypeptide or analogue in a host cell.

9. The polynucleotide of claim 8, wherein said element for expression in a host cell is a promoter.

10. The polynucleotide of any one of claims 5 to 9, wherein said polynucleotide is associated with one or more compounds selected from the group consisting of transfecting agents, stabilizing agents and targeting agents.

11. A vector comprising at least one polynucleotide of any one of claims 5 to 10.

12. The vector of claim 11 comprising at least two different nucleotide sequences encoding at least two polypeptides as defined in claim 3.

13. The vector of claim 11 or 12 which is a plasmid.

14. The vector of claim 11 or 12, which is a viral vector.

15. A host cell comprising a polynucleotide of any one of claims 5 to 10 or a vector of any one of claims 11 to 14.

16. The host cell of claim 15, which is a prokaryotic cell, a yeast cell, or an animal cell, such as a mammalian cell.

17. A composition comprising a polypeptide of any one of claims 1 to 3, an analogue of claim 4, a polynucleotide of any one of claims 5 to 10, a vector of any one of claims 11 to 14, or a host cell of claim 15 or 16 or a combination of two or more of these different compounds.

18. The composition of claim 17, further comprising a pharmaceutical carrier.

19. Use of a polypeptide of any one of claims 1 to 3, of an analogue of claim 4, of a polynucleotide of any one of claims 5 to 10, of a vector of any one of claims 11 to 14, of a host cell of claim 15 or 16 or of a composition of claim 17 for the preparation of a medicament for effecting a CTL response in a subject.

20. A diagnostic composition comprising a polypeptide of any one of claims 1 to 3.

21. A vaccine comprising a polypeptide of any one of claims 1 to 3, an analogue of claim 4, a polynucleotide of any one of claims 5 to 10, a vector of any one of claims 11 to 14 or a host cell of claim 15 or 16, which vaccine is capable of stimulating a MHC class I restricted T cell response directed to an epitope as contained in a polypeptide of any one of claims 1 to 3.

22. The vaccine of claim 21 which comprises an adjuvant or a delivery system, which adjuvant or delivery system stimulates a MHC class I restricted response.

23. A T cell receptor which recognizes an epitope contained in a polypeptide of any one of claims 1 to 3 or a fragment of said T cell receptor which can recognize the epitope.

24. A T cell comprising the T cell receptor of claim 23.

25. The T cell of claim 24, which has been produced by replication *in vitro*.

26. A product that selectively binds a T cell receptor of claim 23.

27. The product of claim 26 which product comprises (a) an HLA molecule, or a fragment thereof, comprising a polypeptide of any one of claims 1 to 3 or an analogue of claim 4 in its peptide binding groove, or (b) an analogue of (a) which is capable of inhibiting the binding of (a) to a T cell receptor of claim 23.

28. A method of identifying a product of claim 26 or 27 comprising contacting a candidate substance with a T cell receptor or fragment of claim 23 and determining whether the candidate substance binds to the T cell receptor or fragment, the binding of the candidate substance to the T cell receptor or fragment indicating that the candidate substance is such a product.

29. A cell comprising a product of claim 26 or 27.

30. A method of identifying a MHC class I restricted T cell response, said method comprising contacting a population of cells comprising MHC class I restricted T cells with:
the polypeptide of any one of claims 1 to 3 or with the analogue of claim 4 under conditions suitable for the presentation of the polypeptide or analogue to the T cells, or
a product of claim 26 or 27 or cells of claim 29; and
determining whether the CD8 T cells recognize the polypeptide, analogue, the product or the cell, recognition by the T cells indicating the presence of a MHC class I restricted T cell response.

31. The method of claim 30, in which the determination of the T cell recognition is done by detecting the expression of a substance by the T cells, the expression of the substance indicating that the T cells have recognized the polypeptide, the analogue, the product or the cell.

32. The method of claim 31, in which the substance which is detected is IFN- γ .

33. A method of diagnosing cancer in a host said method comprising determining the presence or absence in the host of a MHC class I restricted T cell response to a

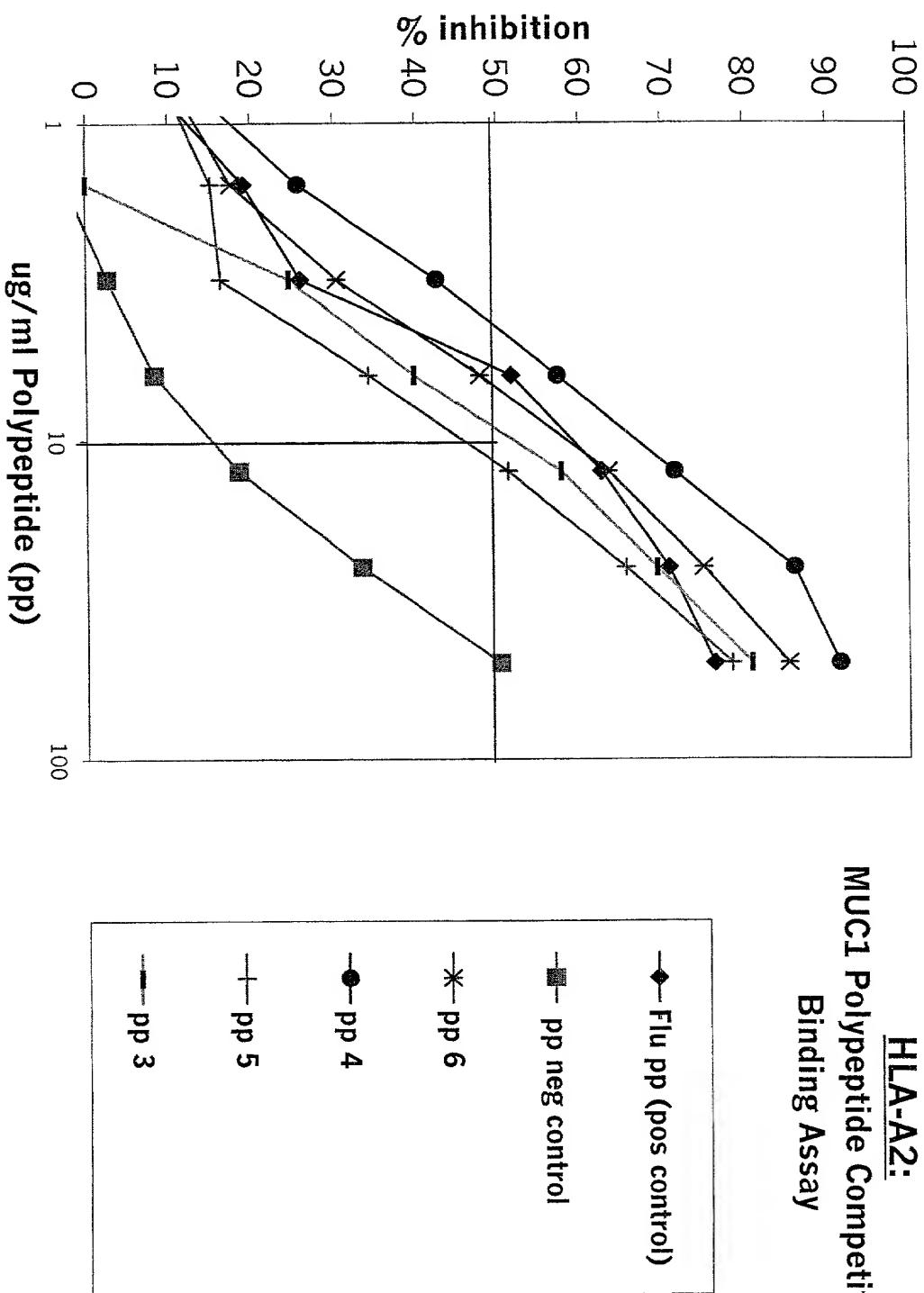
polypeptide of any one of claims 1 to 3, the presence of the MHC class I restricted T cell response indicating that the host has cancer.

34. The method of claim 33, in which the presence or absence of the MHC class I restricted T cell response is determined by the method of any one of claims 30 to 32.
35. A method of causing the replication of MHC class I restricted T cells which recognize an epitope of a cancer cell or an activated T cell, said method comprising contacting a population of cells which comprises MHC class I restricted T cells with the polypeptide of any one of claims 1 to 3 or with the analogue of claim 4 under conditions in which the polypeptide or the analogue are presented to T cells in the population, or with a product of claim 26 or 27 or with a cell of claim 29.
36. A pharmaceutical composition comprising a product of claim 26 or 27, a cell of claim 28, the T cell of claim 24 or 25, or a cell which has been replicated in the method of claim 35.
37. A kit for carrying out a method of any one of claims 30 to 35 comprising a polypeptide of any one of claims 1 to 3, an analogue of claim 4, a polynucleotide of any one of claims 5 to 10, a composition of claim 17 and/or a product of claim 26 or 27.

ABSTRACT

Described are peptides and polypeptides derived from the MUC-1 polypeptide which are able to activate Cytotoxic T Lymphocyte (CTL) response, analogues of such peptides and polypeptides nucleotide sequences encoding such peptides and polypeptides and therapeutic uses thereof. Moreover, indications for selecting appropriate minimal antigenic MUC-1 polypeptides with reference to the HLA-type of the patient to be treated or tested are described.

MUC1 Polypeptide Competition Binding Assay



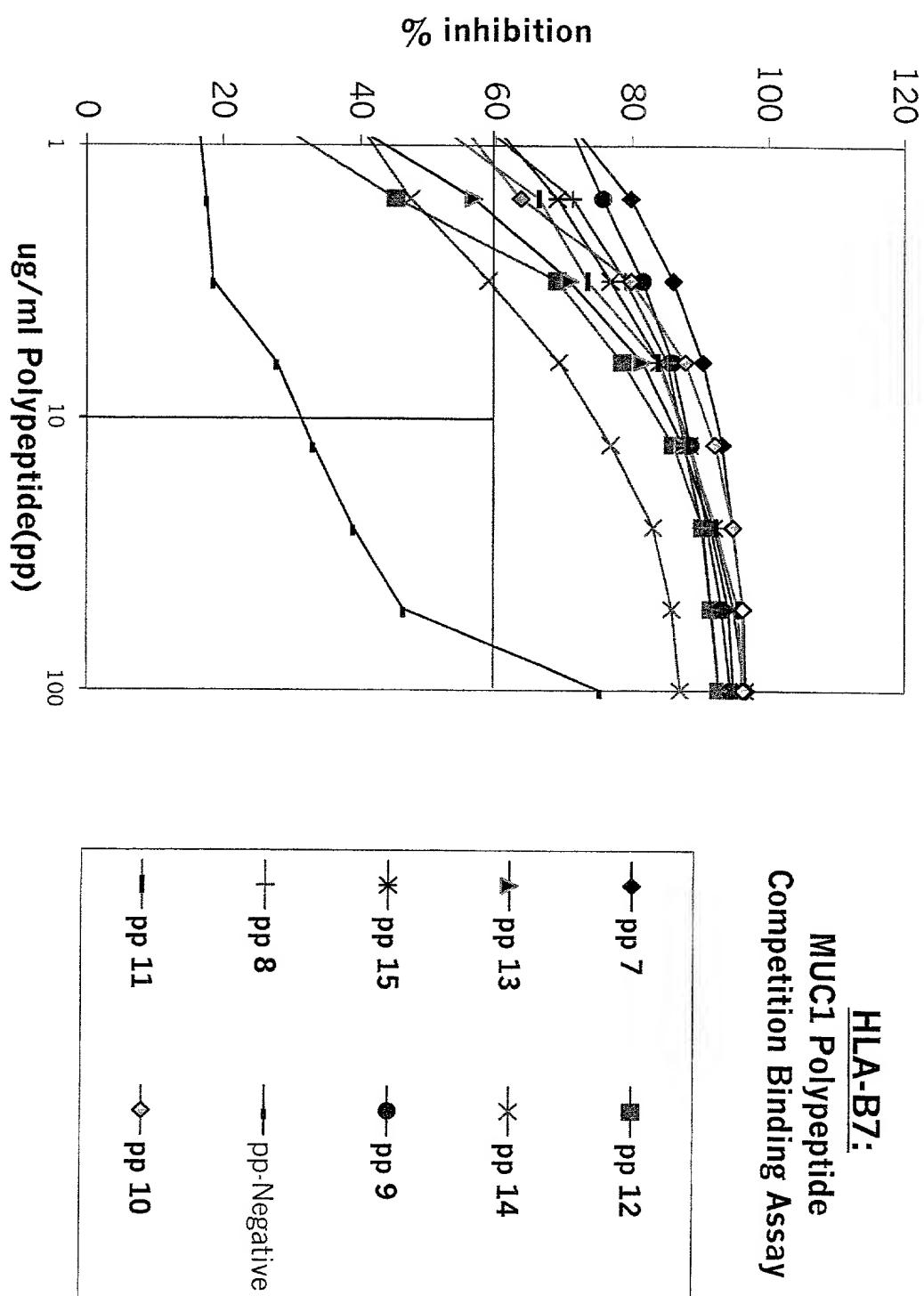
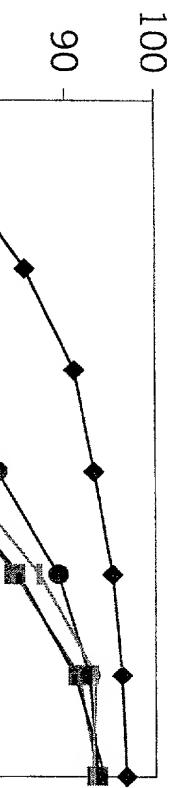


FIGURE 2



HLA-A3:
MUC1 Polypeptide
Competition Binding Assay

FIGURE 3

ug/ml Polypeptide (pp)

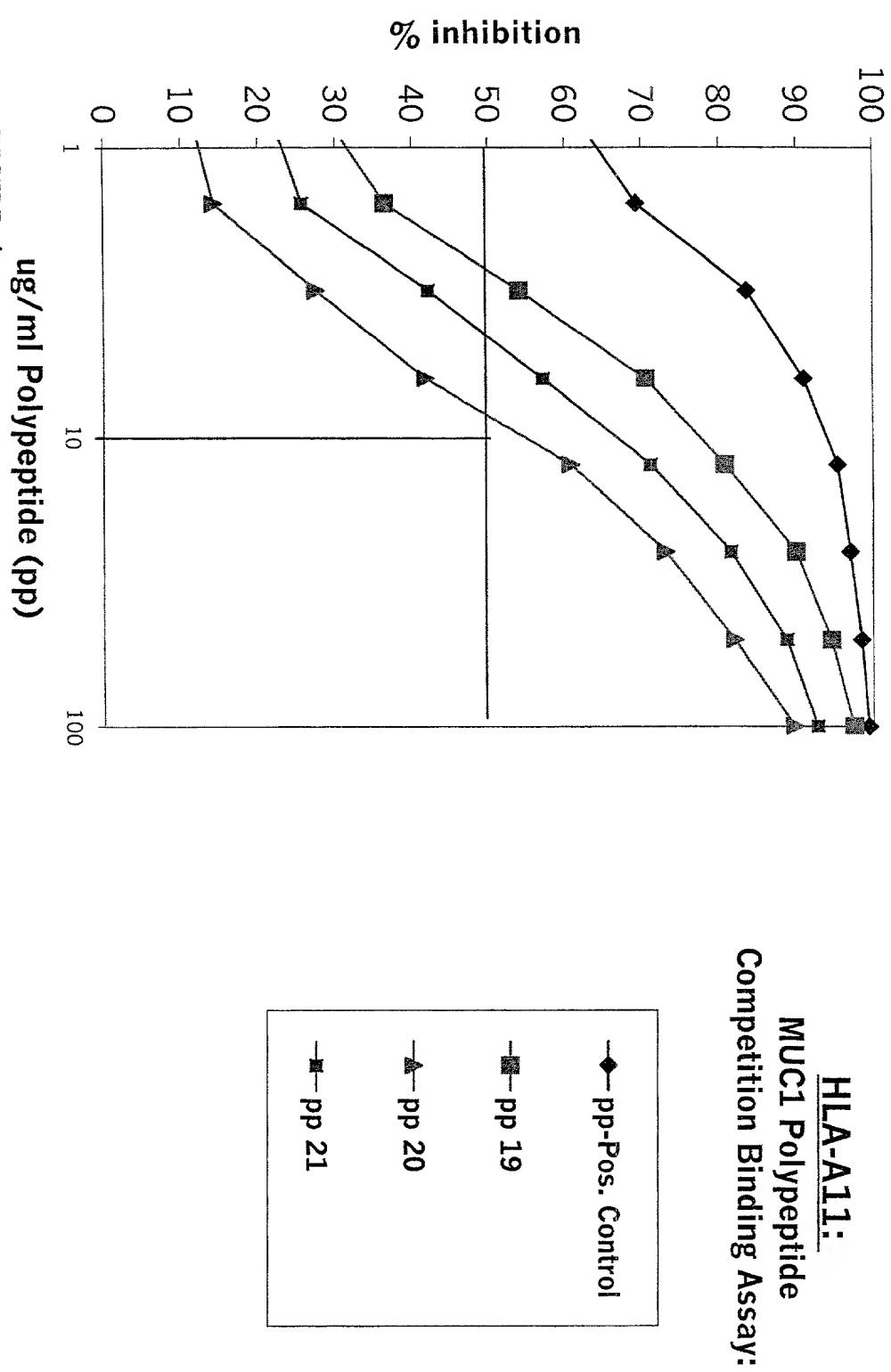


FIGURE 4

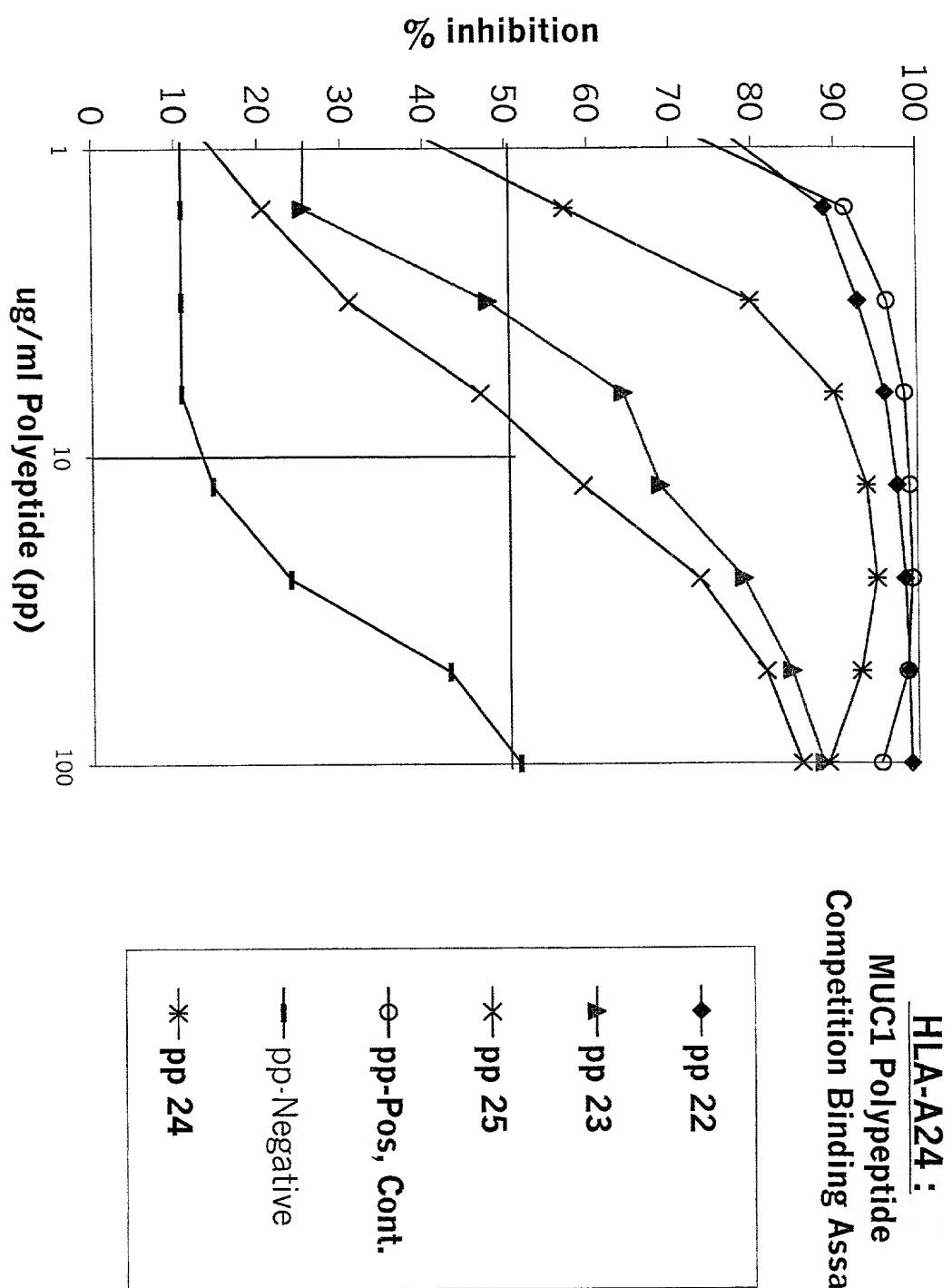


FIGURE 5

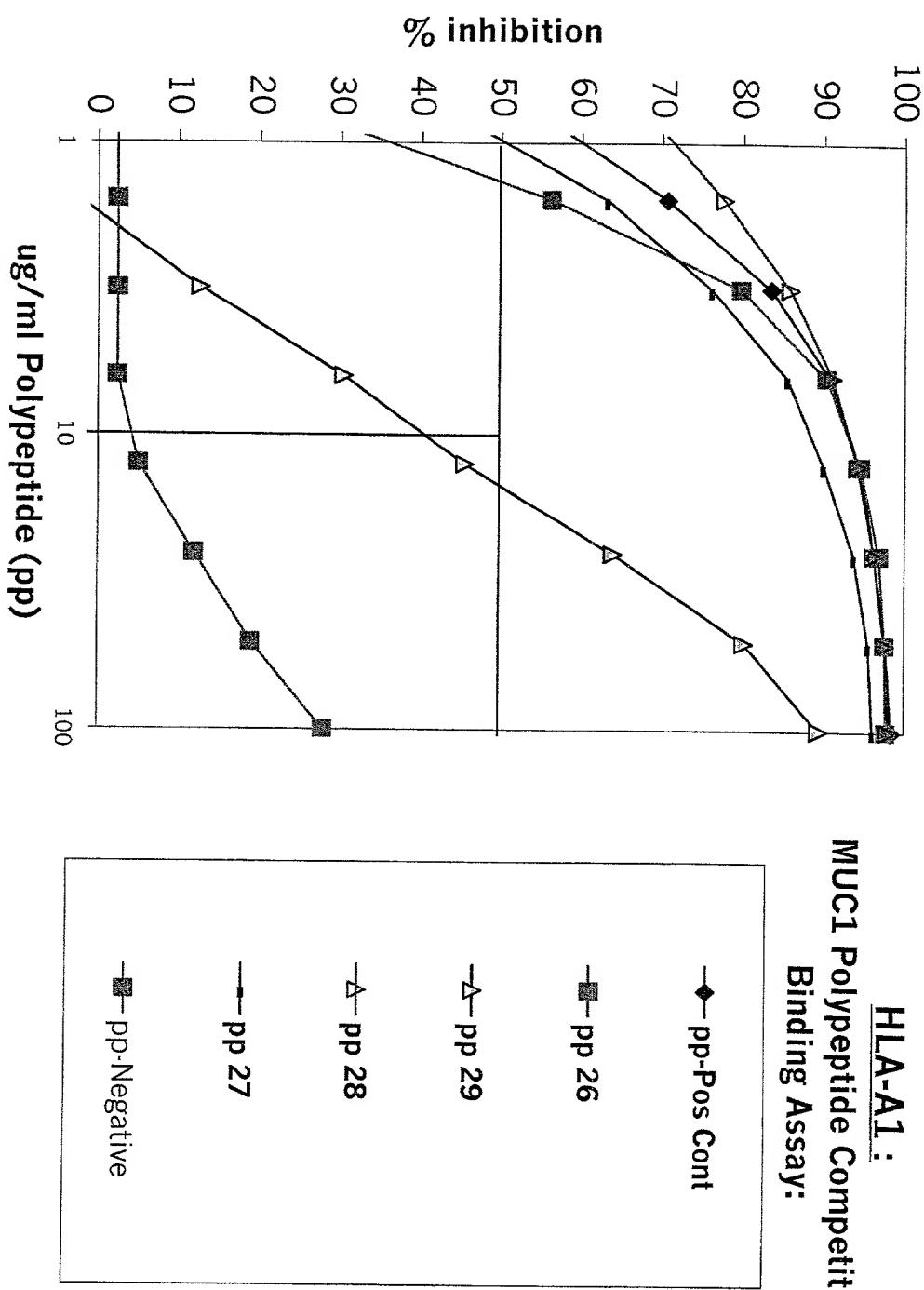


FIGURE 6

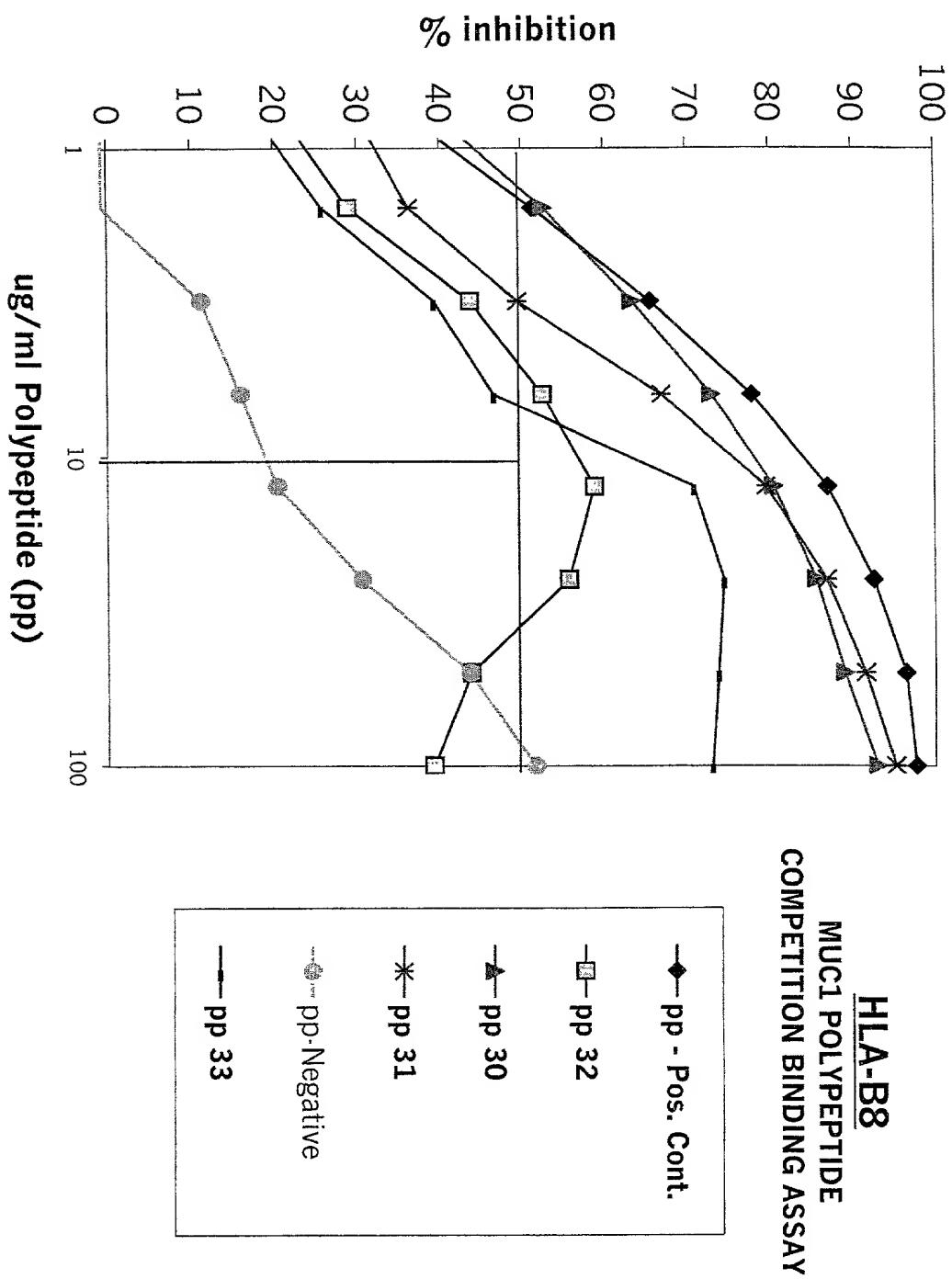


FIGURE 7

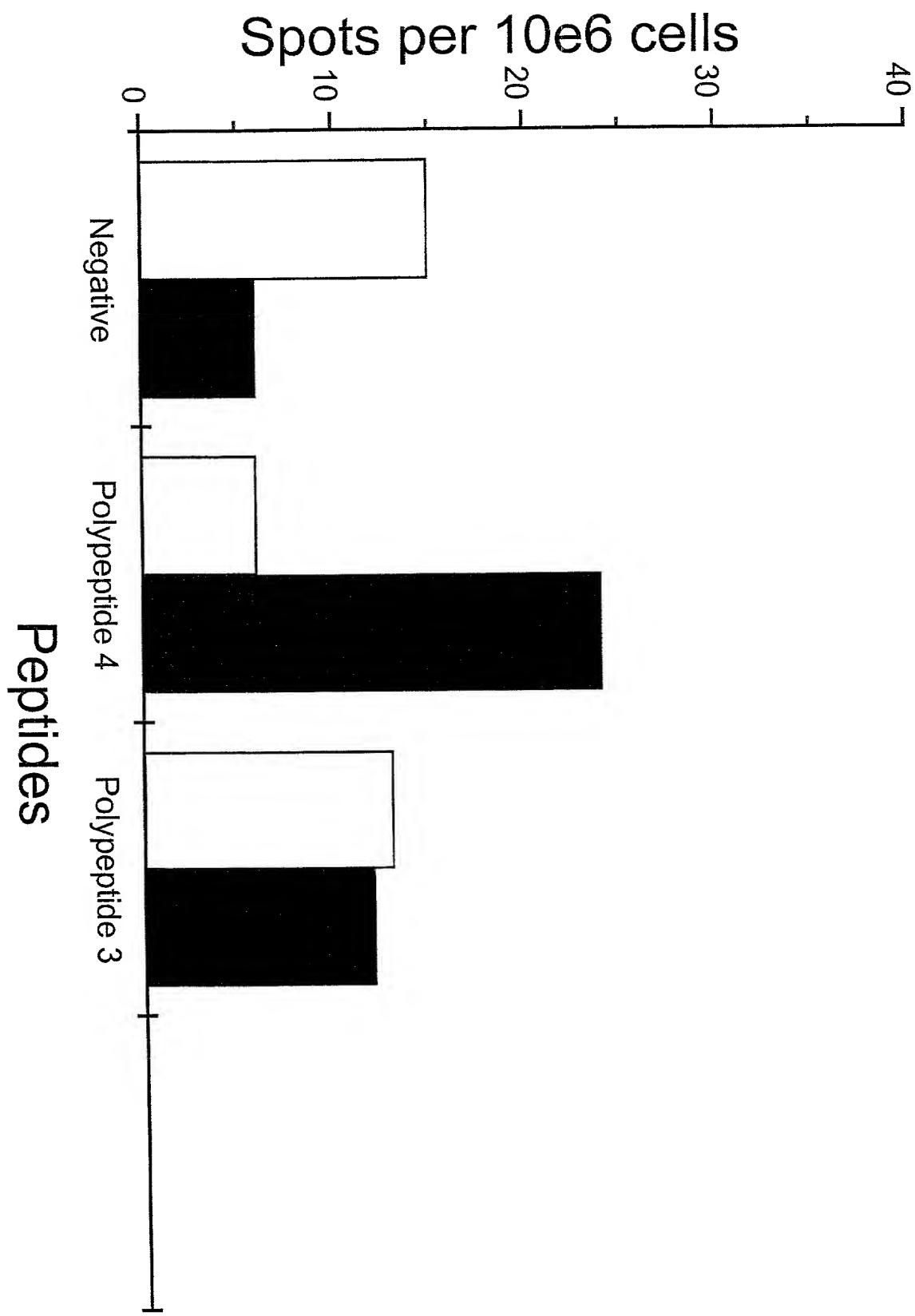


FIGURE 8

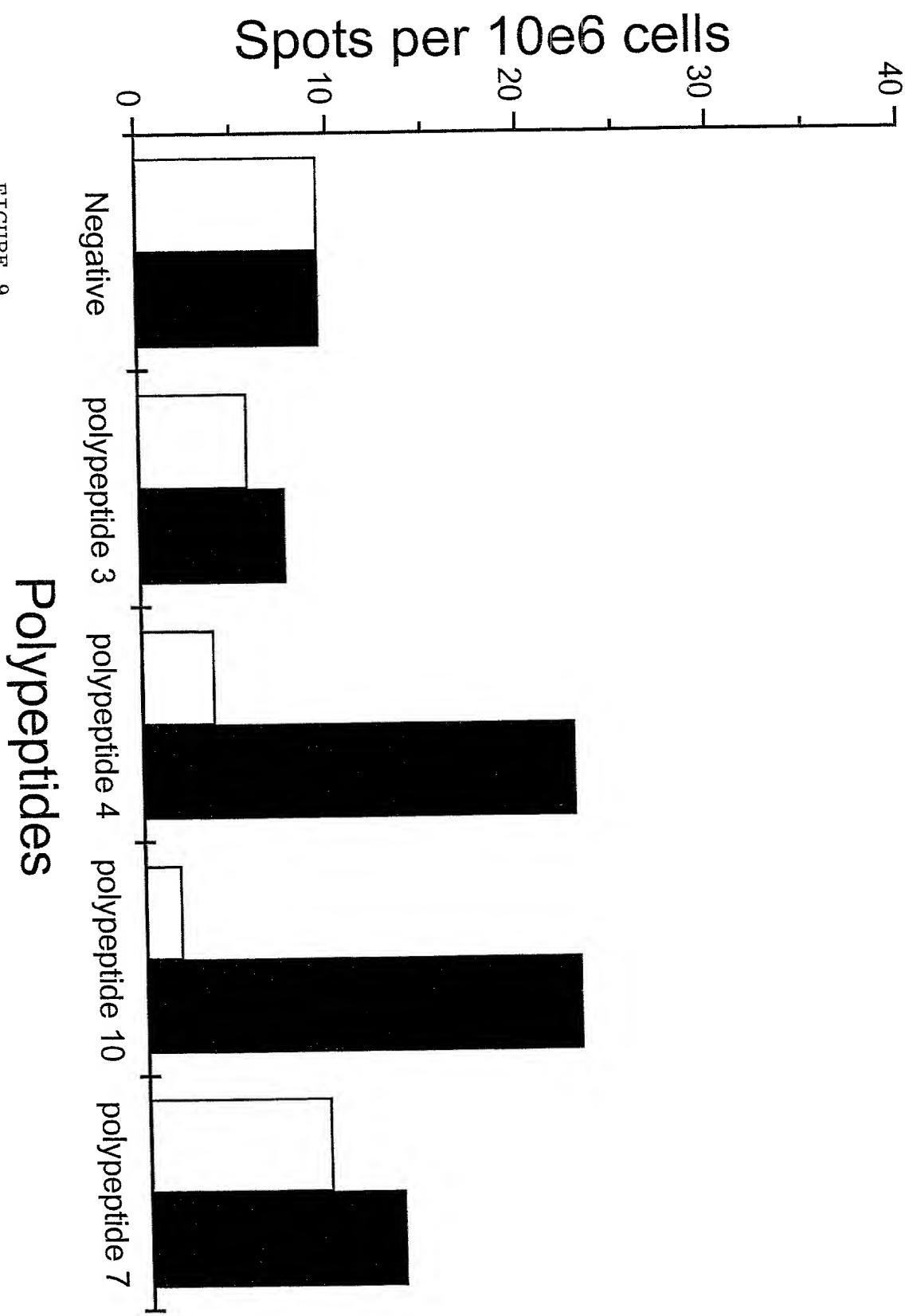


FIGURE 9

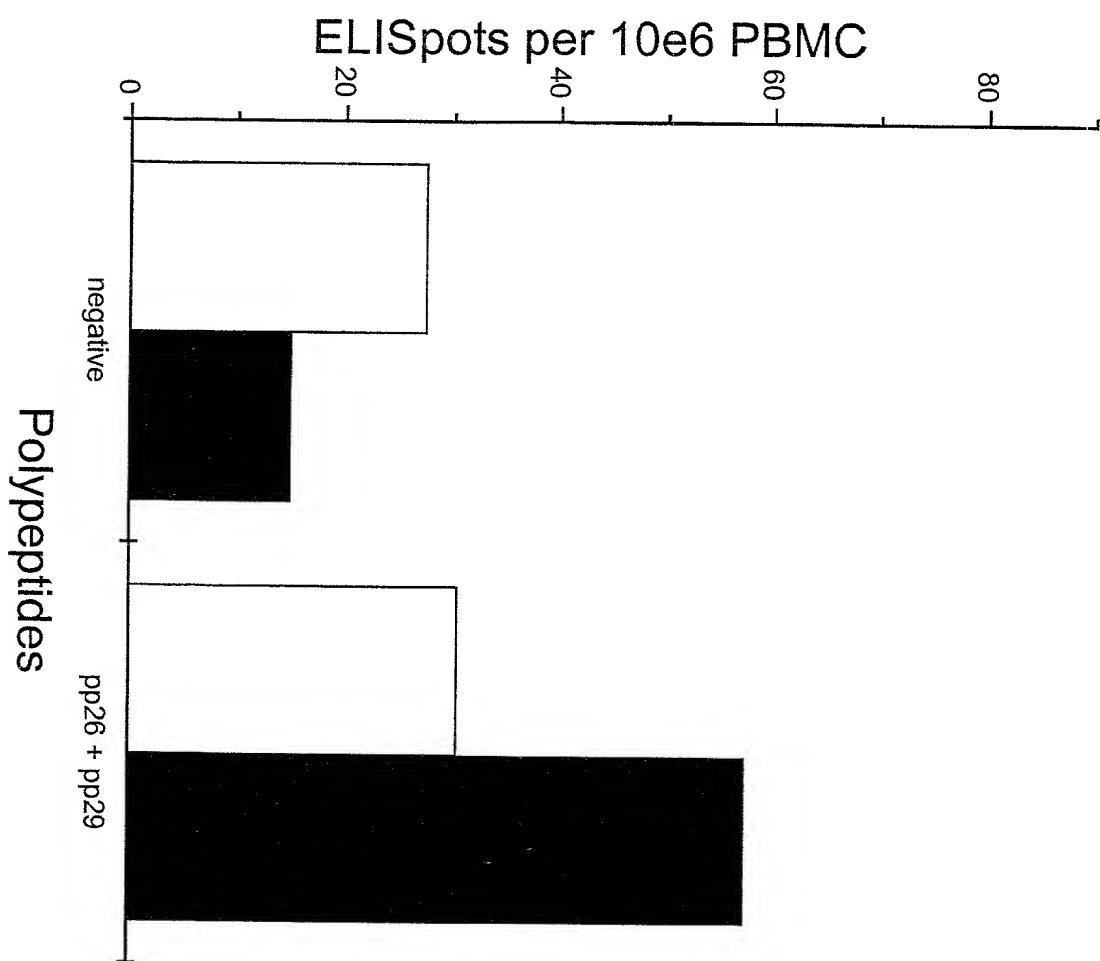
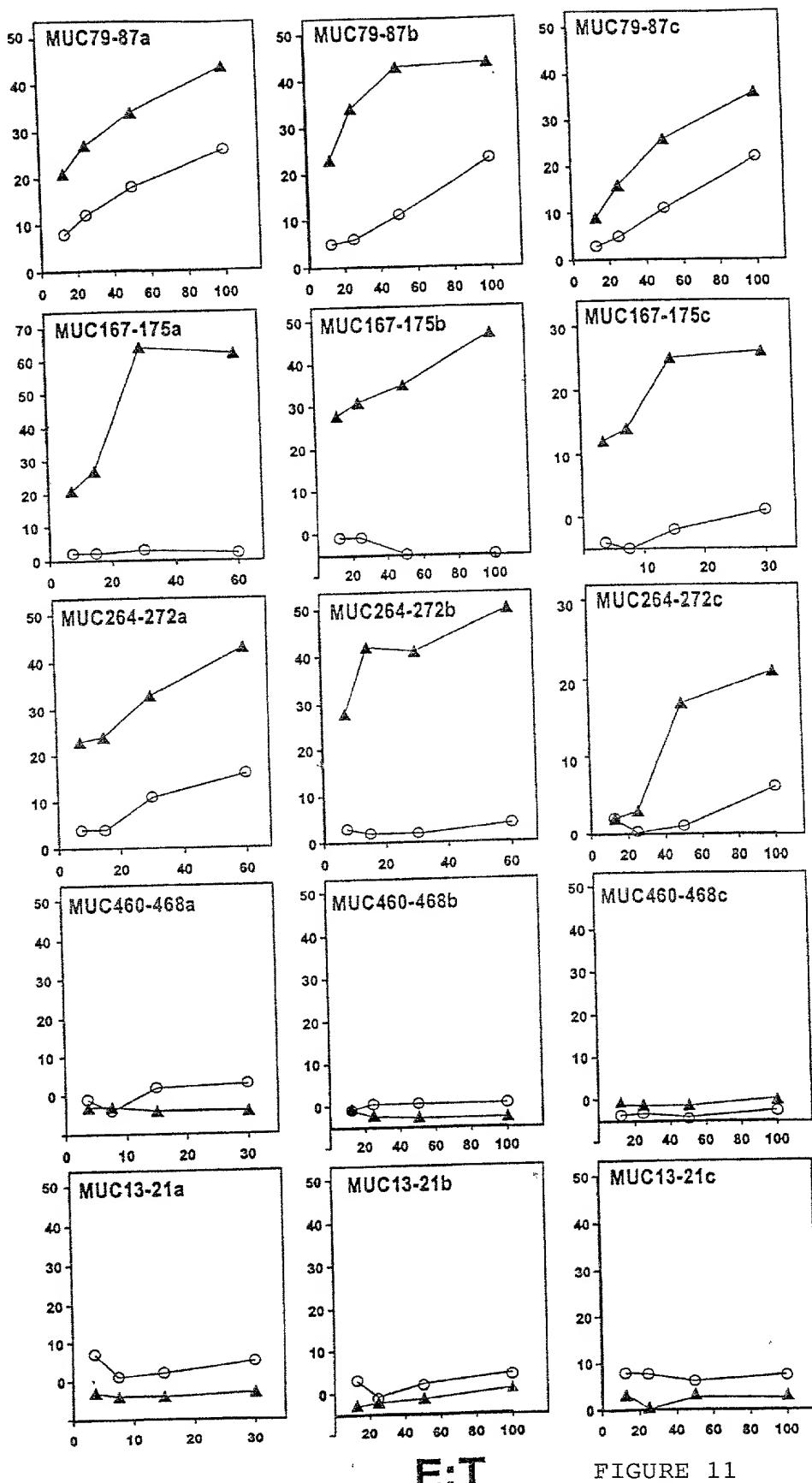


FIGURE 10



Met Thr Pro Gly Thr Gln Ser Pro Phe Phe Leu Leu Leu Leu Leu Thr
 1 5 10 15
 Val Leu Thr Val Val Thr Gly Ser Gly His Ala Ser Ser Thr Pro Gly
 20 25 30
 Gly Glu Lys Glu Thr Ser Ala Thr Gln Arg Ser Ser Val Pro Ser Ser
 35 40 45
 Thr Glu Lys Asn Ala Val Ser Met Thr Ser Ser Val Leu Ser Ser His
 50 55 60
 Ser Pro Gly Ser Gly Ser Ser Thr Thr Gln Gly Gln Asp Val Thr Leu
 65 70 75 80
 Ala Pro Ala Thr Glu Pro Ala Ser Gly Ser Ala Ala Thr Trp Gly Gln
 85 90 95
 Asp Val Thr Ser Val Pro Val Thr Arg Pro Ala Leu Gly Ser Thr Thr
 100 105 110
 Pro Pro Ala His Asp Val Thr Ser Ala Pro Asp Asn Lys Pro Ala Pro
 115 120 125
 Gly Ser Thr Ala Pro Pro Ala His Gly Val Thr Ser Ala Pro Asp Thr
 130 135 140
 Arg Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His Gly Val Thr Ser
 145 150 155 160
 Ala Pro Asp Asn Arg Pro Ala Leu Gly Ser Thr Ala Pro Pro Val His
 165 170 175
 Asn Val Thr Ser Ala Ser Gly Ser Ala Ser Gly Ser Ala Ser Thr Leu
 180 185 190
 Val His Asn Gly Thr Ser Ala Arg Ala Thr Thr Pro Ala Ser Lys
 195 200 205
 Ser Thr Pro Phe Ser Ile Pro Ser His His Ser Asp Thr Pro Thr Thr
 210 215 220
 Leu Ala Ser His Ser Thr Lys Thr Asp Ala Ser Ser Thr His His Ser
 225 230 235 240
 Thr Val Pro Pro Leu Thr Ser Ser Asn His Ser Thr Ser Pro Gln Leu
 245 250 255
 Ser Thr Gly Val Ser Phe Phe Leu Ser Phe His Ile Ser Asn Leu
 260 265 270
 Gln Phe Asn Ser Ser Leu Glu Asp Pro Ser Thr Asp Tyr Tyr Gln Glu
 275 280 285

Figure 12

Leu Gln Arg Asp Ile Ser Glu Met Phe Leu Gln Ile Tyr Lys Gln Gly
290 295 300
Gly Phe Leu Gly Leu Ser Asn Ile Lys Phe Arg Pro Gly Ser Val Val
305 310 315 320
Val Gln Leu Thr Leu Ala Phe Arg Glu Gly Thr Ile Asn Val His Asp
325 330 335
Val Glu Thr Gln Phe Asn Gln Tyr Lys Thr Glu Ala Ala Ser Arg Tyr
340 345 350
Asn Leu Thr Ile Ser Asp Val Ser Val Ser Asp Val Pro Phe Pro Phe
355 360 365
Ser Ala Gln Ser Gly Ala Gly Val Pro Gly Trp Gly Ile Ala Leu Leu
370 375 380
Val Leu Val Cys Val Leu Val Ala Leu Ala Ile Val Tyr Leu Ile Ala
385 390 395 400
Leu Ala Val Cys Gln Cys Arg Arg Lys Asn Tyr Gly Gln Leu Asp Ile
405 410 415
Phe Pro Ala Arg Asp Thr Tyr His Pro Met Ser Glu Tyr Pro Thr Tyr
420 425 430
His Thr His Gly Arg Tyr Val Pro Pro Ser Ser Thr Asp Arg Ser Pro
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Figure 12 (continued)

SEQUENCE LISTING

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Met Thr Pro Gly Thr Gln Ser Pro Phe Phe Leu Leu Leu Leu Thr
1 5 10 15
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Val Leu Thr Val Val Thr Gly Ser Gly His Ala Ser Ser Thr Pro Gly
20 25 30
gga gaa aag gag act tcg gct acc cag aga agt tca gtg ccc agc tct 201
Gly Glu Lys Glu Thr Ser Ala Thr Gln Arg Ser Ser Val Pro Ser Ser
35 40 45
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Thr Glu Lys Asn Ala Val Ser Met Thr Ser Ser Val Leu Ser Ser His
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Ser Pro Gly Ser Gly Ser Thr Thr Gln Gly Gln Asp Val Thr Leu
65 70 75 80
gcc ccg gcc acg gaa cca gct tca ggt tca gct gcc acc tgg gga cag 345
Ala Pro Ala Thr Glu Pro Ala Ser Gly Ser Ala Ala Thr Trp Gly Gln
85 90 95
gat gtc acc tcg gtc cca gtc acc agg cca gcc ctg ggc tcc acc acc 393
Asp Val Thr Ser Val Pro Val Thr Arg Pro Ala Leu Gly Ser Thr Thr
100 105 110
ccg cca gcc cac gat gtc acc tca gcc ccg gac aac aag cca gcc ccg 441
Pro Pro Ala His Asp Val Thr Ser Ala Pro Asp Asn Lys Pro Ala Pro
115 120 125
ggc tcc acc gcc ccc ccg gcc cac ggt gtc acc tcg gcc ccg gac acc 489
Gly Ser Thr Ala Pro Pro Ala His Gly Val Thr Ser Ala Pro Asp Thr
130 135 140

agg ccg ccc ccg ggc tcc acc gcc ccc gcc cac ggt gtc acc tcg Arg Pro Pro Pro Gly Ser Thr Ala Pro Ala Ala His Gly Val Thr Ser 145 150 155 160	537
gcc ccg gac acc agg ccg gcc ccg ggc tcc acc gcc ccc ccg gcc cac Ala Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His 165 170 175	585
ggg gtc acc tcg gcc ccg gac aac agg ccg gcc ttg ggc tcc acc gcc Gly Val Thr Ser Ala Pro Asp Asn Arg Pro Ala Leu Gly Ser Thr Ala 180 185 190	633
cct cca gtc cac aat gtc acc tcg gcc tca ggc tct gca tca ggc tca Pro Pro Val His Asn Val Thr Ser Ala Ser Gly Ser Ala Ser Gly Ser 195 200 205	681
gct tct act ctg gtg cac aac ggc acc tct gcc agg gct acc aca acc Ala Ser Thr Leu Val His Asn Gly Thr Ser Ala Arg Ala Thr Thr Thr 210 215 220	729
cca gcc agc aag agc act cca ccc agc att ccc agc cac cac tct gat Pro Ala Ser Lys Ser Thr Pro Pro Ser Ile Pro Ser His His Ser Asp 225 230 235 240	777
act cct acc acc ctt gcc agc cat agc acc aag act gat gcc agt agc Thr Pro Thr Thr Leu Ala Ser His Ser Thr Lys Thr Asp Ala Ser Ser 245 250 255	825
act cac cat agc acg gta cct ctc acc tcc tcc aat cac agc act Thr His His Ser Thr Val Pro Pro Leu Thr Ser Ser Asn His Ser Thr 260 265 270	873
tct ccc cag ttg tct act ggg gtc tct ttc ttt ttc ctg tct ttt cac Ser Pro Gln Leu Ser Thr Gly Val Ser Phe Phe Phe Leu Ser Phe His 275 280 285	921
att tca aac ctc cag ttt aat tcc tct ctg gaa gat ccc agc acc gac Ile Ser Asn Leu Gln Phe Asn Ser Ser Leu Glu Asp Pro Ser Thr Asp 290 295 300	969
tac tac caa gag ctg cag aga gac att tct gaa atg ttt ttg cag att Tyr Tyr Gln Glu Leu Gln Arg Asp Ile Ser Glu Met Phe Leu Gln Ile 305 310 315 320	1017
tat aaa caa ggg ggt ttt ctg ggc ctc tcc aat att aag ttc agg cca Tyr Lys Gln Gly Gly Phe Leu Gly Leu Ser Asn Ile Lys Phe Arg Pro 325 330 335	1065
gga tct gtg gtg gta caa ttg act ctg gcc ttc cga gaa ggt acc atc Gly Ser Val Val Gln Leu Thr Leu Ala Phe Arg Glu Gly Thr Ile 340 345 350	1113
aat gtc cac gac gtg gag aca cag ttc aat cag tat aaa acg gaa gca Asn Val His Asp Val Glu Thr Gln Phe Asn Gln Tyr Lys Thr Glu Ala 355 360 365	1161
gcc tct cga tat aac ctg acg atc tca gac gtc agc gtg agt cat gtg Ala Ser Arg Tyr Asn Leu Thr Ile Ser Asp Val Ser Val Ser His Val 370 375 380	1209
cca ttt cct ttc tct gcc cag tct ggg gct ggg gtg cca ggc tgg ggc Pro Phe Pro Phe Ser Ala Gln Ser Gly Ala Gly Val Pro Gly Trp Gly 385 390 395 400	1257

atc gcg ctg ctg gtg ctg gtc tgt gtt ctg gtt gcg ctg gcc att gtc Ile Ala Leu Leu Val Leu Val Cys Val Leu Val Ala Leu Ala Ile Val 405	410	415	1305	
tat ctc att gcc ttg gct gtc tgt cag tgc cgc cga aag aac tac ggg Tyr Leu Ile Ala Leu Ala Val Cys Gln Cys Arg Arg Lys Asn Tyr Gly 420	425	430	1353	
cag ctg gac atc ttt cca gcc cggtt gat acc tac cat cct atg agc gag Gln Leu Asp Ile Phe Pro Ala Arg Asp Thr Tyr His Pro Met Ser Glu 435	440	445	1401	
tac ccc acc tac cac acc cat ggg cgc tat gtg ccc cct agc agt acc Tyr Pro Thr Tyr His Thr His Gly Arg Tyr Val Pro Pro Ser Ser Thr 450	455	460	1449	
gat cgt agc ccc tat gag aag gtt tct gca ggt aat ggt ggc agc agc Asp Arg Ser Pro Tyr Glu Lys Val Ser Ala Gly Asn Gly Gly Ser Ser 465	470	475	480	1497
ctc tct tac aca aac cca gca gtg gca gcc act tct gcc aac ttg Leu Ser Tyr Thr Asn Pro Ala Val Ala Ala Thr Ser Ala Asn Leu 485	490	495	1542	
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<p>Thr Glu Lys Asn Ala Val Ser Met Thr Ser Ser Val Leu Ser Ser His 50 55 60</p>				
<p>Ser Pro Gly Ser Gly Ser Ser Thr Thr Gln Gly Gln Asp Val Thr Leu 65 70 75 80</p>				
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<p>Asp Val Thr Ser Val Pro Val Thr Arg Pro Ala Leu Gly Ser Thr Thr 100 105 110</p>				
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<p>Arg Pro Pro Pro Gly Ser Thr Ala Pro Ala Ala His Gly Val Thr Ser 145 150 155 160</p>				

Ala Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His
 165 170 175
 Gly Val Thr Ser Ala Pro Asp Asn Arg Pro Ala Leu Gly Ser Thr Ala
 180 185 190
 Pro Pro Val His Asn Val Thr Ser Ala Ser Gly Ser Ala Ser Gly Ser
 195 200 205
 Ala Ser Thr Leu Val His Asn Gly Thr Ser Ala Arg Ala Thr Thr Thr
 210 215 220
 Pro Ala Ser Lys Ser Thr Pro Pro Ser Ile Pro Ser His His Ser Asp
 225 230 235 240
 Thr Pro Thr Thr Leu Ala Ser His Ser Thr Lys Thr Asp Ala Ser Ser
 245 250 255
 Thr His His Ser Thr Val Pro Pro Leu Thr Ser Ser Asn His Ser Thr
 260 265 270
 Ser Pro Gln Leu Ser Thr Gly Val Ser Phe Phe Phe Leu Ser Phe His
 275 280 285
 Ile Ser Asn Leu Gln Phe Asn Ser Ser Leu Glu Asp Pro Ser Thr Asp
 290 295 300
 Tyr Tyr Gln Glu Leu Gln Arg Asp Ile Ser Glu Met Phe Leu Gln Ile
 305 310 315 320
 Tyr Lys Gln Gly Gly Phe Leu Gly Leu Ser Asn Ile Lys Phe Arg Pro
 325 330 335
 Gly Ser Val Val Val Gln Leu Thr Leu Ala Phe Arg Glu Gly Thr Ile
 340 345 350
 Asn Val His Asp Val Glu Thr Gln Phe Asn Gln Tyr Lys Thr Glu Ala
 355 360 365
 Ala Ser Arg Tyr Asn Leu Thr Ile Ser Asp Val Ser Val Ser His Val
 370 375 380
 Pro Phe Pro Phe Ser Ala Gln Ser Gly Ala Gly Val Pro Gly Trp Gly
 385 390 395 400
 Ile Ala Leu Leu Val Leu Val Cys Val Leu Val Ala Leu Ala Ile Val
 405 410 415
 Tyr Leu Ile Ala Leu Ala Val Cys Gln Cys Arg Arg Lys Asn Tyr Gly
 420 425 430
 Gln Leu Asp Ile Phe Pro Ala Arg Asp Thr Tyr His Pro Met Ser Glu
 435 440 445
 Tyr Pro Thr Tyr His Thr His Gly Arg Tyr Val Pro Pro Ser Ser Thr
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Ser Ala Gly Asn Gly Gly Ser Ser Leu
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 35 40 45

Thr Glu Lys Asn Ala Val Ser Met Thr Ser Ser Val Leu Ser Ser His
 50 55 60

Ser Pro Gly Ser Gly Ser Ser Thr Thr Gln Gly Gln Asp Val Thr Leu
 65 70 75 80

Ala Pro Ala Thr Glu Pro Ala Ser Gly Ser Ala Ala Thr Trp Gly Gln
 85 90 95

Asp Val Thr Ser Val Pro Val Thr Arg Pro Ala Leu Gly Ser Thr Thr
 100 105 110

Pro Pro Ala His Asp Val Thr Ser Ala Pro Asp Asn Lys Pro Ala Pro
 115 120 125

Gly Ser Thr Ala Pro Pro Ala His Gly Val Thr Ser Ala Pro Asp Thr
 130 135 140

Arg Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His Gly Val Thr Ser
 145 150 155 160

Ala Pro Asp Asn Arg Pro Ala Leu Gly Ser Thr Ala Pro Pro Val His
 165 170 175

Asn Val Thr Ser Ala Ser Gly Ser Ala Ser Gly Ser Ala Ser Thr Leu
 180 185 190

Val His Asn Gly Thr Ser Ala Arg Ala Thr Thr Pro Ala Ser Lys

195

200

205

Ser Thr Pro Phe Ser Ile Pro Ser His His Ser Asp Thr Pro Thr Thr
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Leu Ala Ser His Ser Thr Lys Thr Asp Ala Ser Ser Thr His His Ser
 225 230 235 240

Thr Val Pro Pro Leu Thr Ser Ser Asn His Ser Thr Ser Pro Gln Leu
 245 250 255

Ser Thr Gly Val Ser Phe Phe Leu Ser Phe His Ile Ser Asn Leu
 260 265 270

Gln Phe Asn Ser Ser Leu Glu Asp Pro Ser Thr Asp Tyr Tyr Gln Glu
 275 280 285

Leu Gln Arg Asp Ile Ser Glu Met Phe Leu Gln Ile Tyr Lys Gln Gly
 290 295 300

Gly Phe Leu Gly Leu Ser Asn Ile Lys Phe Arg Pro Gly Ser Val Val
 305 310 315 320

Val Gln Leu Thr Leu Ala Phe Arg Glu Gly Thr Ile Asn Val His Asp
 325 330 335

Val Glu Thr Gln Phe Asn Gln Tyr Lys Thr Glu Ala Ala Ser Arg Tyr
 340 345 350

Asn Leu Thr Ile Ser Asp Val Ser Val Ser Asp Val Pro Phe Pro Phe
 355 360 365

Ser Ala Gln Ser Gly Ala Gly Val Pro Gly Trp Gly Ile Ala Leu Leu
 370 375 380

Val Leu Val Cys Val Leu Val Ala Leu Ala Ile Val Tyr Leu Ile Ala
 385 390 395 400

Leu Ala Val Cys Gln Cys Arg Arg Lys Asn Tyr Gly Gln Leu Asp Ile
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Phe Pro Ala Arg Asp Thr Tyr His Pro Met Ser Glu Tyr Pro Thr Tyr
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His Thr His Gly Arg Tyr Val Pro Pro Ser Ser Thr Asp Arg Ser Pro
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Tyr Glu Lys Val Ser Ala Gly Asn Gly Ser Ser Leu Ser Tyr Thr
 450 455 460

Asn Pro Ala Val Ala Ala Thr Ser Ala Asn Leu
 465 470 475